Transdermal delivery of a buprenorphine/naltrexone combination for the treatment of polydrug abuse

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Award date:
2016

Awarding institution:
University of Bath

Transdermal delivery of a buprenorphine/naltrexone combination for the treatment of polydrug abuse

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A thesis submitted for the degree of Doctor of Philosophy

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October 2015

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Acknowledgements

I would like to thank my supervisors, Begona Delgado-Charro and Chris Bailey, who have been extremely supportive and helpful throughout my PhD. I would also like to thank Stephen Husbands, Richard Guy and Alistair Taverner for their invaluable input.

Abstract

This thesis investigated a buprenorphine (BUP) and naltrexone (NTX) combination therapy for the prevention of relapse to heroin and cocaine. Delivery of the combination by transdermal iontophoresis was also explored.

In the first half of the thesis, experiments were carried out using Sprague Dawley rats; an assay of analgesia (the tail withdrawal assay) and a behavioural assay (conditioned place preference; CPP) were employed. It was found that 1.0 mg/kg of NTX (but not 0.3 mg/kg NTX) blocked the analgesic and rewarding properties of 0.3 mg/kg BUP. Interestingly, 0.3 mg/kg BUP with 3.0 mg/kg NTX, but not the same dose of NTX alone, was aversive. Using a CPP-extinction-reinstatement method, it was shown that the combination (0.3 mg/kg BUP and 1.0 mg/kg NTX), administered 10 minutes before a priming dose, prevented reinstatement of both morphine- and cocaine-seeking.

In the second half of the thesis, iontophoresis (0.3 or 0.4 mA/cm^2) was used to transport BUP and NTX across the skin; experiments were carried out using excised pig skin in side-bi-side glass cells. NTX transport was efficient and consistent with the standard mechanisms (electrorepulsion and electroosmosis) of iontophoresis. BUP was less efficiently delivered and accumulated in the skin; the size of the BUP skin depot increased with pH and with the concentration of BUP in the donor solution. BUP's presence in the skin reduced convective solvent flow (indicated by a neutral marker) which had a negative impact on its flux, and also on that of NTX.

The BUP/NTX combination has real potential as a relapse prevention therapy, and it is particularly exciting that it seems to be efficacious against cocaine, as treatments for cocaine dependence are desperately needed. Delivery of a therapeutically relevant amount of the combination by transdermal iontophoresis is achievable, but careful investigation is required to understand the atypical behaviour of BUP.

List of abbreviations

ACM	acetaminophen
BUP	buprenorphine hydrochloride
Cl	clearance
CPP	conditioned place preference
DP	drug-paired compartment
EO	electroosmosis
ER	electrorepulsion
GABA	gamma-amino butyric acid
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography mass spectroscopy
MF	molar fraction
NOP	Nociceptin/Orphanin FQ receptor
norBUP	norbuprenorphine
NTX	naltrexone hydrochloride
6ß-NTX	6ß-naltrexol
PBS	phosphate buffered saline
PI	pulsed iotophoresis
RP	reversing polarity
SEM	standard error of the mean
SI	standard iontophoresis
SP	saline-paired compartment
SPE	solid phase extraction
TN	transport number

Chapter 1. Introduction

1. Treatment of addiction

Addiction is widespread and costly to the individual and to society. Globally, around 200 million people use psychoactive substances (not including alcohol) each year (WHO 2015). In the UK, around 1600 people die per year as a result of using illegal drugs (Office for National Statistics), and around 1600 people are living with HIV as a result of intravenous drug use (Health Protection Agency).

It is reported that 42 % of patients with substance abuse problems never achieve a sustained recovery (defined as one year with no symptoms) (Dennis *et al*. 2005). Treatment is difficult at least in part because a large number of drug users have psychiatric conditions (Dennis *et al.* 2005) and because concurrent use of multiple drugs of abuse is common (Leri *et al*. 2003).

Heroin and crack cocaine are the two most harmful illegal drugs of abuse to the user as well as to others (Nutt *et al*. 2010). Treatment for dependence upon heroin and crack cocaine is the focus of this thesis.

Characteristics of addiction

The action of drugs of abuse is often described as a 'hijacking' of the natural reward systems of the brain. These reward systems exist because they reinforce fundamental behaviours such as eating, drinking, mating, and bonding with offspring. The effects of drugs of abuse are powerful because they do not simply mimic the natural reward, they far exceed it (Kalivas & O'Brien 2008). The effects of different addictive substances (alcohol, nicotine, amphetamines, cocaine, opioids) are mediated by distinct pharmacological mechanisms, but many behavioural commonalities can be observed. Indeed, clinical diagnosis of addiction does not distinguish between substances involved. Addiction is characterised by the following points (Redish *et al*. 2008; Badiani *et al*. 2011):

- Some people are predisposed to addiction
- People often first use drugs of abuse to self-medicate
- Poly-drug use is common (such as alcohol with nicotine, or cocaine with heroin)
- The motivation behind drug use changes from using the drug because it makes you feel good ('positive reinforcement') to because without the drug you feel terrible ('negative reinforcement')

- Drug use evolves from a choice to a compulsion (this is associated with bad decision-making)
- Drug use continues despite the experience no longer being pleasurable (liking changes to wanting)
- Drug use continues despite negative consequences (such as significant detrimental effect on the user's personal life, work, health)
- The association between objects/people/places and the drug becomes strengthened
- There is a high propensity to relapse (to resume drug use even after long periods of abstinence)

It is generally believed that for all drugs of abuse dependence is related to an increase in levels of dopamine in the nucleus accumbens (Kreek *et al.* 2012). This commonality between different classes of drugs of abuse means that it is theoretically possible for a single pharmacological treatment to be effective against all drugs of abuse.

Current strategies for pharmacological treatment of addiction

Many relapse prevention therapies mediate their effect by blockade or substitution. These strategies can be illustrated by differing approaches to smoking cessation. Bupropion, available in tablet form, provides an example of the blockade mechanism. It does not elicit nicotine-like effects, and blocks the pleasurable effects of nicotine if the patient smokes. Nicotine, available as patches or gum, acts by substitution. The nicotine diminishes withdrawal symptoms, reducing (or eliminating) the need to smoke and therefore avoiding the associated health problems.

Some relapse prevention therapies have aspects of both blockade and substitution. For example, varenicline, which is sold as tablets for smoking cessation, blocks the effects of nicotine, but itself gives a low level nicotine-like effect.

Current pharmacological treatments for heroin

Methadone is the mainstay of heroin treatment in the UK. It acts primarily as a substitution therapy, by activating the same receptors as heroin, and thus reducing withdrawal symptoms (Farid *et al*. 2008), allowing patients to regain some control of their lives (Nutt & Lingford-Hughes 2008). Methadone treatment has some shortcomings. Methadone is itself a very addictive drug, and can be fatal in overdose, by respiratory depression. Furthermore, it does not eliminate heroin use, and a patient on methadone is not protected from a fatal heroin

overdose. Lastly, many fatalities of children have occurred following accidental ingestion of methadone (Milroy & Forrest 2000).

Naltrexone (NTX) is prescribed to heroin-dependent patients as an abstinence-promoter. Although NTX is safe, and effective at blocking the euphoric effects of heroin (Schuh *et al.* 1999; Comer *et al.* 2006), treatment success is hindered by low compliance (Kirchmayer *et al*. 2002).

A third treatment option for heroin-dependence is buprenorphine (BUP). It acts both as a substitution therapy, similar to methadone, and as a blockade therapy, similar to NTX (Greenwald *et al.* 2003). BUP can be rewarding (Greenwald *et al.* 2007; Marquez *et al.* 2007) therefore concerns exist over abuse liability.

As mentioned, this thesis focuses not only on the treatment of dependence on heroin but also on crack cocaine. Unfortunately, whilst the symptoms associated with ceasing long-term use of cocaine (such as sleeplessness and anxiety) can be treated, no treatment to reduce the likelihood of relapse is currently available.

Euphoria and reward

The brain areas involved in both acute and chronic effects of rewarding substances include the amygdala, hippocampus, hypothalamus, several regions of the frontal cortex, the VTA (ventral tegmental area), and the NAc (nucleus accumbens). The pathway from the VTA in the midbrain to the NAc in the limbic forebrain (the mesolimbic dopamine pathway) is considered particularly important, and an increase in dopamine release in the NAc appears to be a common effect of both drugs of abuse and of natural rewards such as food and sex (Berridge & Robinson 1998).

Figure 1.1 illustrates that despite diverse pharmacology, all drugs of abuse (including opiates and cocaine) have convergent effects at the mesolimbic dopamine pathway, resulting in a common outcome of increased dopaminergic transmission in the NAc (Di Chiara & Imperato 1988).

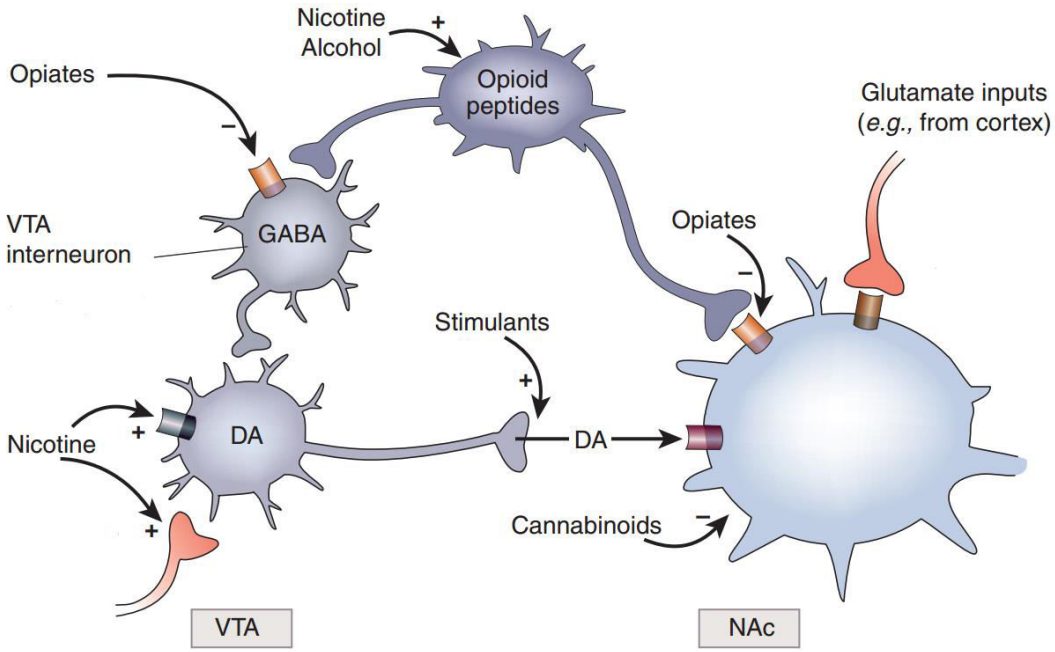

Figure 1.1: Simplified diagram of the acute actions of several drugs of abuse on the ventral tegmental area (VTA) and the nucleus accumbens (NAc). Cocaine is represented here under the description 'stimulants'. Adapted from Nestler (2005), and reproduced with kind permission of Nature Publishing Group.

Opiates

The opioid system comprises three classical receptors: mu, kappa, and delta, plus the more recently characterised NOP (Nociceptin/Orphanin FQ). NOP is also known as ORL-1 (Opioid Receptor-Like). The primary ligands of the mu, kappa and delta receptors are the endogenous peptides β-endorphin, dynorphin, and met- and leu-enkephalin (Mucha *et al*. 1985). The endogenous ligand of NOP is nociceptin (Meunier *et al*. 1995; Reinscheid *et al*. 1995).

The opioid receptors are G-protein-coupled receptors, and are broadly expressed throughout the central and peripheral nervous system (Le Merrer *et al*. 2009). Binding sites of mu, kappa and delta overlap in most brain structures, although higher expression of one receptor type may be seen in some regions.

The mu receptor is well studied, partly because many analgesics, such as morphine, mediate their therapeutic effects (and indeed their side-effects such as respiratory depression) via the mu receptor. Agonism of the mu receptor can also produce euphoria (see next section).

Agonism of the kappa receptor also has analgesic effects. However, unlike mu agonism it does not cause respiratory depression, and instead of euphoria can elicit dysphoria

(negative mood state). Antagonism of the kappa receptor can have an anti-depressant or anti-stress effect (Almatroudi *et al.* 2015). The kappa/dynorphin system and the mu system oppose and balance each other to maintain a baseline normal mood state (Shippenberg & Herz 1986).

The importance of the delta receptor in the treatment of addiction is the least well studied and remains unclear. The effect of agonism of the NOP receptor by the endogenous peptide nociceptin is complex, but as its name suggests, it has some pain-producing effects (Lutfy & Maidment 2000).

Activation of the opioid receptors causes inhibition of adenylate cyclase, hyperpolarisation due to the opening of potassium channels, and inhibition of transmitter release due to the inhibition of the opening of calcium channels. Opioids bind to opioid receptors on GABAergic interneurons in the VTA. This results in a disinhibition of VTA dopaminergic neurones that project to the NAc, and an increase in dopamine levels. Opioids also bind to opioid receptors on neurons in the NAc itself.

Cocaine

Cocaine increases levels of dopamine, noradrenaline and 5-HT in the synapse in the central and peripheral nervous system (Phillips *et al.* 2014). This is generally attributed to the binding of cocaine to the respective reuptake transporters of dopamine, noradrenaline and 5-HT (DAT, NET and SERT) and consequent blocking of their activity delaying clearance of those neurotransmitters from the synapse back into the pre-synaptic neurone. A mechanism whereby dopamine transport by DAT is actually inverted has also been more recently described (Badiani *et al.* 2011; Heal *et al.* 2014). Either way, an increased level of extracellular dopamine is probably the most important mediator for cocaine's stimulating and euphoric properties (Heal *et al*. 2014).

Changes in the brain due to chronic drug use

Physiological changes occur during chronic drug use (Wise & Koob 2014). Sustained high levels of mu agonism (due to chronic heroin use, for example) cause endogenous systems to shift toward normal pre-drug levels (classical homeostasis). The mu system is down-regulated and the kappa/dynorphin system is up-regulated (Bruchas *et al.* 2010). This up-regulation of the kappa/dynorphin system is also observed following chronic cocaine use (Shippenberg 2009). When a drug user abstains from using drugs, agonism of the mu-opioid receptor is dramatically reduced, but the kappa/dynorphin system is still in "overdrive"; the resulting imbalance causes dysphoria. Ceasing chronic use of drugs of abuse can therefore

leave patients in a very unpleasant physical and psychological state (Rothman *et al*. 2000). Furthermore, during periods of abstinence, patients' pre-existing pathologies (such as depression or anxiety) may emerge. This state of mind leaves patients vulnerable to relapse triggered by stress, people/objects/places strongly associated with previous drug-taking, or by a small intake of the drug of abuse (Aguilar *et al.* 2009).

The mood state of patients is critical to retention in treatment programs, and therefore critical to likelihood of relapse (Rothman *et al*. 2000). As abstinent patients may be dysphoric due to kappa overdrive, administration of a kappa antagonist is a rational treatment strategy (Carroll & Carlezon 2013). Unfortunately, no selective kappa antagonists are currently licensed. Rothman *et al*. (2000) suggested that delivering two currently licensed compounds, BUP and NTX, together was a way of achieving the desired pharmacological profile of kappa antagonism plus very low level mu agonism. This combination is further investigated in this thesis.

Naltrexone

NTX is an antagonist at the mu and the kappa receptors, and binds with comparable affinity at both these receptors (Toll *et al.* 1998). The low compliance documented for NTX may be related to the fact that it provides no pleasure (Kirchmayer *et al*. 2002). Furthermore, as an antagonist of the mu receptor it is likely to block natural rewards (Mucha *et al*. 1985) and exacerbate the patient's dysphoria. The idea that mu antagonists can block endogenous rewards appears to be supported by preclinical work (Mucha *et al.* 1985) that showed that removing the brain region of rats that produce β-endorphin eliminated the observed aversive effect of naloxone.

Poor adherence to NTX is a serious concern; if the patient does not take the medication for a day or two, blockade of heroin will not be effective, because NTX has a half-life of around 24 hours (Nutt & Lingford-Hughes 2008). Lastly, it is interesting to note that NTX is also used for treatment of alcohol-dependence. It is suggested that in the event of a lapse the pleasure from drinking is lessened therefore the chance of triggering a relapse is reduced (Pettinati *et al*. 2006).

Buprenorphine

BUP is a partial agonist of the mu receptor, and an antagonist of the kappa receptor (Husbands 2013). As a partial agonist at the mu receptor, BUP can act as an agonist or an antagonist, depending on the dose and the specific test conditions (Schuh *et al.* 1999). As an agonist, BUP can be described as a substitution therapy. It follows that it can cause a

positive mood state, and can be rewarding (Greenwald *et al.* 2007; Marquez *et al.* 2007). BUP has high affinity but low efficacy at the mu receptor, and can therefore act to block the actions of other mu agonists, such as hydromorphone (Schuh *et al*. 1999) and heroin (Law *et al.* 2004).

As previously stated, BUP is a partial agonist at the mu receptor. This means that it can never elicit the same response as full opioid agonists (such as morphine, for example) even at high doses. Consequently, compared to full agonists, BUP exhibits a ceiling effect for respiratory depression, and for its rewarding properties (Lewis 1985). Although the ceiling effect on respiratory depression does mean that BUP alone is very safe, intravenous injection in conjunction with other drugs such as benzodiazapenes may be harmful (Nutt & Lingford-Hughes 2008).

BUP and NTX combination

This approach could be considered as a NTX therapy in which BUP is included to make it more attractive to patients; this is critical to encourage individuals to remain in treatment (Nutt 2010). Alternatively, the combination can be viewed as a BUP therapy in which NTX is included to limit abuse liability. Inclusion of sufficient NTX should be able to limit BUP's mu-opioid receptor agonism (Dum & Herz 1981; McAleer *et al.* 2003) thus increasing regulatory acceptability, and feasibility of its use in cocaine addicts. Overall it is desired that the combination of BUP and NTX be neither rewarding nor aversive.

Though not mentioned in Rothman's original rationale for the combination (2000), BUP is also a partial agonist of the NOP receptor (Huang *et al.* 2001). Selective NOP agonists are neither rewarding nor aversive (Le Pen *et al.* 2002) and, although the mechanism is poorly understood, they have been shown in rodents to oppose the effects of both cocaine and morphine (Kotlinska *et al.* 2002; Sakoori & Murphy 2004).

Rothman presented the BUP/NTX combination primarily as a treatment for heroin-dependent patients. McCann (2008) also championed a BUP/NTX combination for relapse prevention, but with a slightly different rationale. There is some evidence that BUP might reduce cocaine use (Kosten *et al.* 1989) but it might not be appropriate to administer even a partial mu agonist to opioid-naïve cocaine-dependent patients. Inclusion of NTX in the combination would address that concern. Lastly, McCann pointed out that the majority of cocaine-dependent patients also abuse alcohol, and NTX is already licensed for alcohol dependence. Overall, a mixed very low efficacy mu receptor agonist, kappa receptor antagonist, NOP receptor agonist is a desirable target as a novel anti-addiction therapy.

Note that the rationale for combining BUP with the mu antagonist naloxone in the analgesic sublingual tablet Suboxone® is completely different from the rationale for combining BUP with NTX for relapse prevention here described. In Suboxone®, naloxone is included as an anti-abuse strategy. Due to low bioavailability, naloxone has no pharmacological effect if the product is used as indicated, but if the tablet is crushed and injected, the naloxone blocks the potentially rewarding effects of the BUP. Naloxone is not well suited to long-term treatment of heroin dependence due to its relatively short duration of action (van Dorp *et al*. 2006) and is more commonly used to treat opiate overdose.

Clinical evidence for a BUP/NTX combination as a relapse prevention therapy

A small study involving drug users was carried out to test whether a BUP/NTX combination was effective as a relapse prevention treatment (Rothman *et al.* 2000). In these studies, NTX and BUP were administered as oral tablets and sublingual tablets respectively. The results were encouraging; a significant reduction of both heroin and cocaine use was demonstrated. A second, slightly larger study was therefore carried out (Gerra *et al*. 2006), in which the BUP/NTX therapy was directly compared with NTX alone. The effect of the combination was superior to that of NTX alone. Retention rates were higher in the combination group (22 out of 30 completers compared to 12 out of 30 completers for the NTX-only group). Patients taking the combination showed reduced drug use (figure 1.2), and reduced scores for craving and psychiatric symptoms including depression and anxiety.

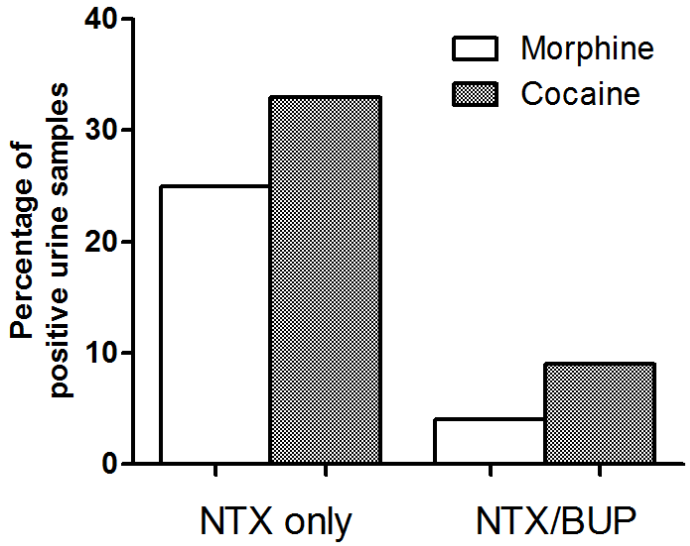

Figure 1.2: Treatment with NTX/BUP resulted in lower rates of positive urine samples than treatment with NTX only ($p < 0.05$) after 12 weeks of treatment. Drawn from data from Gerra *et al*. (2006).

Both Rothman and Gerra attribute the success of the BUP/NTX combination primarily to kappa antagonism restoring the balance between the mu and kappa systems, thus

restoring mood. Gerra concedes that a small amount of mu agonism from BUP might also aid outcomes. Regardless of the mechanism responsible, overall the combination probably works because it reduces the need to self-medicate (Gerra *et al*. 2006).

Effect of BUP/NTX on cocaine use

Interestingly, a reduction in cocaine use in the combination group was observed in both clinical studies (Rothman *et al*. 2000; Gerra *et al*. 2006). This effect has also been observed when BUP (Kosten *et al*. 1989) and NTX (Schmitz *et al.* 2001) are administered separately. If the rewarding effects of cocaine are mediated by dopamine, it is not immediately obvious why treatment with opioids should be effective. However it has been reported that human polymorphisms at the mu receptor influence predisposition to cocaine addiction (Kreek *et al.* 2012; Yuferov *et al*. 2010), and preclinical evidence indicates that cocaine use results in region-specific activation of both the mu and kappa systems (Yoo *et al*. 2012).

The reduction in cocaine use observed in the Rothman (2000) and Gerra (2006) studies could be a result of antagonism of mu receptors attenuating the positive reinforcing effects of cocaine (Bilsky *et al.* 1992), or a result of antagonism of kappa receptors conferring stress resilience (Redila & Chavkin 2008), or a consequence of more generalized anti-addictive effects of agonism of the NOP receptor (Shoblock *et al.* 2005; Kuzmin *et al.* 2007). Alternatively, the observed reduction in cocaine use in these two clinical studies could simply be a consequence of reduction in heroin use; these two drugs of abuse are often used by addicts to 'complement' each other (Leri *et al*. 2003). Therefore, the impact of BUP/NTX on the effects of cocaine in a controlled experiment has been assessed in Chapter 2 of this thesis.

2. **Delivery strategy for buprenorphine and naltrexone**

Formulation of BUP and NTX together in a single medicine is likely to be fundamental to the success of this combination therapy. As BUP can be rewarding when not blocked by NTX, physical incorporation of the two compounds together is necessary to minimise the chance of abuse or diversion of BUP. Furthermore, adherence is a particular concern in this patient group, and would be facilitated with a simpler therapy.

Oral tablets, though cheap and convenient, are not a straightforward option for co-formulation of BUP and NTX. NTX can be administered orally, but BUP has low oral bioavailability due to significant first pass effect (McQuay & Moore 1995). Conversely, BUP can be administered as a sublingual tablet (or sublingual film), but NTX has low

bioavailability by the sublingual route (Lewis & Lloyd-Jones 1987). Therefore, in the clinical studies already mentioned (Rothman *et al.* 2000; Gerra *et al.* 2006), BUP and NTX were administered sublingually and orally respectively. A similar situation is seen for a clinical trial of BUP and NTX for reduction of cocaine use currently underway (planned study design described in Mooney *et al.* 2013), where NTX is being administered as a long-acting parenteral (Vivitrol®) and BUP is being delivered as a sublingual tablet (Suboxone).

Co-formulation of BUP and NTX in an injectable depot or a surgically-inserted depot (with each depot lasting several months, for example) has obvious advantages related to patient compliance (Sigmon *et al*. 2006), and NTX implants have achieved some success in patients compared to oral tablets (Carreno *et al.* 2003). However, there are several drawbacks to depots. Administration of the drug/s cannot be easily discontinued in the event of adverse reactions. Specifically, there is concern over how to provide acute (opioid) pain relief to patients with an NTX implant. Lastly, no titration of dose can be carried out (until the next depot is administered).

Rationale for the transdermal route for a BUP/NTX therapy

In this thesis, the administration route selected for co-delivery of BUP and NTX was the transdermal route. Transdermal delivery can be used to achieve a continuous input of drug. It is envisaged that BUP/NTX patches would be replaced every few days.

General advantages of the transdermal route include avoidance of first-pass metabolism and of food effects, less frequent dosing for drugs with very short half-lives, ability to discontinue the medication immediately, and suitability for patients who cannot swallow or who are vomiting (Cleary 1993; Berner & John 1994). The possible advantages of delivery of NTX by the transdermal route have been previously discussed. Oral NTX is associated with gastrointestinal disturbances, which would potentially be avoided by delivering NTX transdermally (Paudel *et al*. 2005; Valiveti *et al.* 2005). The low patient retention rates reported for NTX (Kirchmayer *et al*. 2002) may be due, at least in part, to its side effects (nausea, headache, anxiety). It is thought that the side effects of NTX are caused by its primary metabolite, 6-beta-naltrexol (6ß-NTX) (King *et al.* 1997), though the mechanism is unknown. Transdermal delivery would address this in two separate ways. Firstly, a slow steady input of NTX would avoid plasma peaks of 6ß-NTX. Secondly, the ratio of the parent drug to the metabolite is larger when NTX is delivered transdermally compared to orally (Wermeling *et al*. 2008), the authors suggest that this might be due to avoidance of presystemic first-pass metabolism of NTX when it is delivered transdermally.

Transdermal patches for delivery of BUP alone have already been commercialised (Transtec, Norspan, Butrans), for provision of analgesia. The rationale for this administration route for BUP included steadier plasma concentrations than can be achieved with the sublingual route (Lanier *et al.* 2007). In a relapse prevention therapy, a slow input rate of BUP is particularly important. The speed with which a drug enters the brain is very important to its subjective effects (Gorelick 1998; Samaha & Robinson 2005). A critical aspect of substitution therapy is that the therapeutic drug effect occurs more slowly than for the drug of abuse. For example, the slow onset of methadone (thought to be a consequence of both oral consumption and slow binding to receptors) is critical to its use (Bailey & Husbands 2014). The slow input of BUP that can be expected from a transdermal patch means that the treatment itself is unlikely to be rewarding.

Specific advantages of formulating a BUP/NTX combination as a transdermal patch are as follows. It is likely that a relatively constant ratio of NTX:BUP plasma levels would be more easily achieved using continuous drug release from a patch than from the concurrent release of BUP and NTX from daily administration of tablets or films. Secondly, BUP incorporated into an adhesive patch (in the presence of NTX) would be relatively tamper-proof. Despite these advantages, to the author's knowledge, no attempt has previously been made to deliver NTX and BUP together via the transdermal route.

The skin as a site for drug delivery

Three distinct tissue layers lie between the surface of the skin and the cutaneous blood supply (a distance of 200 μm or so; Bronaugh & Stewart 1986): the stratum corneum, the viable epidermis, and the dermis (see figure 1.3).

The stratum corneum is a tough, flexible, waxy layer around 10 μm thick (Kligman 1964), and is the layer of the skin that hinders water loss. The corneocytes (the cells of the stratum corneum) are flattened, relatively impermeable, dead cells filled mainly with keratin, largely devoid of lipids, stacked in interdigitated columns. Each corneocyte is around 1 μm thick, and ~ 40 μm wide (Prausnitz *et al.* 2004). The corneocytes are embedded in a matrix of extracellular, highly ordered lipid bilayers composed of cholesterol, ceramides and free fatty acids.

Below the stratum corneum is the viable epidermis. This is a cellular, aqueous environment, around 50-100 μm thick (van Smeden *et al*. 2014). Underneath the epidermis is the dermis, which is a largely acellular, aqueous, gel-like connective tissue that contains mainly collagen in a matrix of glycosaminoglycans. The capillaries of the cutaneous microcirculation extend

to the top of the dermis, and follow the undulations of the boundary between the dermis and the epidermis.

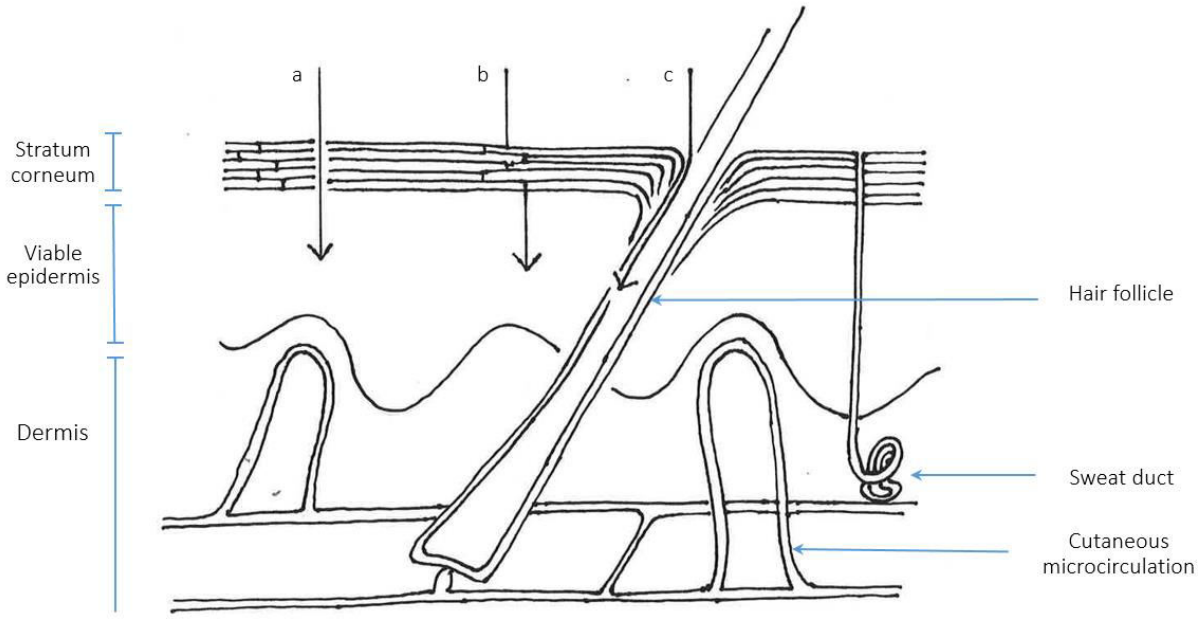

Figure 1.3: Schematic representation of a cross section through human skin. **a**, **b** and **c** show theoretical routes for skin permeation; a = intracellular, b = intercellular, c = appendageal (i.e., via a hair follicle or sweat gland). Redrawn from Prausnitz *et al*. 2004, and Jepps *et al*. 2013.

Although this thesis is concerned with iontophoretic delivery, a brief description of passive permeation is included for completeness and is focussed on transdermal delivery of drugs for systemic action, i.e., penetrating the skin to reach the cutaneous microcirculation.

It is widely accepted that the stratum corneum is very effective at hindering the movement of drug molecules from the surface of the skin into the body. However, despite extensive research, a clear consensus on the route that drugs take across this barrier has not been reached. There are three theoretical options (Scheuplein 2013). Drug molecules could take the shortest route through the skin (shown as **a** in figure 1.3). This would involve repeatedly passing alternately through the corneocytes and the lipid cement around them, and is therefore considered by many to be thermodynamically unfeasible (Cullander 1992; Guy 2013). Drug molecules could pass through the stratum corneum, but only move through the lipid cement (shown as **b** in figure 1.3). This is often referred to as the 'tortuous' pathway because to move around 10 to 15 layers of the flat wide corneocytes, the drug molecules may travel up to 500 µm (Hadgraft 2004) to cross the ~10 µm thick stratum corneum. For exponents of this pathway, the unique geometry of the cells of the stratum corneum helps to explain its excellent barrier properties (Potts & Francoeur 1991).

Lastly, drug molecules could enter the hair follicles or sweat ducts (shown as **c** in figure 1.2). The sweat duct route is often largely ignored, because they occupy such a small area of the skin surface (Edwards & Langer 1994), and furthermore, are either closed, or there is an outward movement of sweat, which would hinder drug ingress (Robson 1988). Hair follicles also occupy a small area of the skin surface (hair follicles on the chest and upper arm occupy ~0.2% of area, Otberg *et al.* 2004). To be clear, the hair follicle route does not circumvent the stratum corneum; the follicles are lined with stratum corneum, albeit thinner (Jepps *et al.* 2013). Obviously, these three routes across the skin are not mutually exclusive, and a drug may travel via all of these routes, at different rates, dependent on the physicochemical properties of the drug, the condition of the skin, and perhaps the stage of drug permeation (Keister & Kasting 1986; Scheuplein 2013).

The influence of the physicochemical properties of compounds on their rate of passive permeation through the skin has been well studied (Naik *et al.* 2000; Wiedersberg & Guy 2014). Not surprisingly, all things being equal, smaller molecules permeate faster than larger molecules, and, as a rule of thumb, molecules over 500 Da are not considered good candidates for transdermal delivery. Molecules can cross the skin easier in the unionised form. A balance of lipophilicity and hydrophilicity is important; a decent water solubility (≥ 1mg/ml) and a logP between 2 and 3 are optimal. Some water solubility is desirable because transdermal formulations may be formulated as a hydrogel, for example. The balance of lipophilicity and hydrophilicity is important because a drug molecule must permeate the lipid barrier of the stratum corneum, before moving into the aqueous environment of the viable tissue (Guy 2013).

The transdermal products currently available are used for quite varied clinical indications, for example: testosterone gel (testosterone replacement), rotigotine patches (Parkinson's disease), oxybutynin patches (overactive bladder), fentanyl patches (analgesia), scopolamine patches (travel sickness), and nicotine patches (smoking cessation). Not only do these compounds display sufficient permeability through the skin, but they have another unifying feature: they are all relatively potent. Passive permeation across the skin is slow, therefore only drug compounds with therapeutic plasma concentrations of a few ng/ml are likely to be successfully delivered via the transdermal route (Kalia *et al.* 2004).

Target transdermal fluxes for a BUP and NTX relapse prevention therapy

A minimum required transdermal flux (input rate) can be calculated from the clearance of the drug and the therapeutic plasma concentrations required at the steady-state, if known (Equation 1.1).

Equation 1.1:

Clearance (L/h) x plasma concentration (µg/L) = Required steady-state flux (µg/h)

The optimal plasma concentrations of BUP and NTX for the treatment of opioid dependence are not yet precisely known, but there are studies in the literature that indicate required therapeutic plasma levels of the two drugs separately. Table 1.1 shows the predicted required input rates for BUP and NTX, calculated from the predicted therapeutic plasma concentrations and on literature values of clearance. Note that this calculation assumes 100% bioavailability. In reality, bioavailability for the transdermal route may be less than 100% because some drug may be metabolised in the skin, or sequestered in the outer layers of the skin and never reach its target (Berner & John 1994).

Unfortunately, in the studies in man by Rothman (2000) and Gerra (2006), plasma concentrations of NTX and BUP were not measured. Thus table 1.1 shows therapeutic concentration values from other sources. For NTX, the predicted therapeutic plasma concentration comes from a handful of studies in man where NTX's ability to block the effects of heroin was assessed (Farid *et al.* 2008). For BUP, the predicted therapeutic plasma concentrations come from studies measuring withdrawal symptoms in opioid-dependent volunteers (Kuhlman *et al*. 1998; Lanier *et al*. 2008) and from a study measuring extent of blockade of hydromorphone challenges (Sigmon *et al.* 2006). The predictions in table 1.1 are based on the therapeutic plasma concentrations of BUP and NTX when administered separately, so the plasma concentrations required as part of a *combination* therapy may well be lower.

The selection of a patch size of 50 cm^2 for the calculation of predicted fluxes was arbitrary, but was considered to represent an acceptable size for the patient.

Table 1.1: Transdermal passive fluxes of BUP and NTX required to keep a therapeutic steady-state concentration to treat opioid dependence. Values were predicted using equation 1.1 ($J_{trans} = C_{ss} \times Cl$) and reported clearance values.

	Clearance in man (L/h)	Therapeutic plasma concentration (μg/L)	Required steady-state flux (μg/h)	Required area normalised flux for a 50 cm^2 patch (μg/cm^2/h)
NTX	211 (Lictko 1981) 201 (Wall *et al.* 1981) 157 (Wall *et al.* 1984)	2 (Farid *et al*. 2008)	379	7.6
BUP	76.5 (Bullingham *et al.* 1980) 76.8 (Kuhlman *et al*. 1996)	0.7 (Kuhlman *et al*. 1998) 0.74 (Sigmon *et al*. 2006) 0.60 (Lanier *et al*. 2008)	54	1.1

To put these target fluxes into context by comparison with some commercial (passive) products, the flux of estradiol is 0.09 – 0.42 μg/cm^2/hr, of granisetron (to treat chemotherapy-induced nausea and vomiting) is 2.5 μg/cm^2/hr, and of methylphenidate (for treatment of attention deficit hyperactivity disorder) is 88 μg/cm^2/hr. So, the required fluxes of BUP and NTX are within the range attainable for other drugs. It is now considered whether they are feasible for BUP and NTX. Table 1.2 shows the fluxes of NTX *in vitro* reported in previous work.

Table 1.2: Previous *in vitro* studies on transdermal delivery of NTX.
[a]values estimated from measured flux of the prodrugs, assuming complete conversion into NTX.

Author	Approach	Details	Maximum flux achieved (μg/h/cm^2)
Hammell *et al.* 2004	NTX prodrugs	Human skin	2.8[a]
Hammell *et al*. 2005	NTX prodrugs	Human skin	2.3[a]
Paudel *et al.* 2005	Delivering the active metabolite of NTX	Guinea pig and human skin. 75 % propylene glycol in the donor solution	4.2 & 1.8
Valiveti *et al*. 2005	NTX prodrugs	Hairless guinea pig skin, saturated solution in light mineral oil	2.8[a]
Wermeling *et al.* 2008	Microneedles, NTX as HCl salt	Human skin. Similar fluxes observed *in vivo*.	14.7
Banks *et al.* 2008	Microneedles, NTX as base and as HCl salt, and the active metabolite of NTX	Guinea pig and human skin. 75 % propylene glycol in the donor solution	13.3
Vaddi *et al.* 2009	NTX prodrugs	Human skin	1.4[a]
Milewski *et al*. 2013	Microneedles, NTX as HCl salt	Yucatan pig skin	1.8
Ghosh *et al.* 2013	Microneedles, NTX as base	Yucatan pig skin	16.4
Ghosh *et al.* 2014	Microneedles, naltrexone diclofenac codrugs	Yucatan pig skin	3.8

It can be seen that conventional passive fluxes of NTX are lower than the predicted flux required for our application. NTX does not cross intact skin easily by passive diffusion, most likely due to its relative hydrophilicity (Paudel *et al*. 2005), having a logP of 1.7, and a water solubility of > 60 mg/ml, and 378 Da in size. The structure of NTX (and BUP) is shown in figure 1.4.

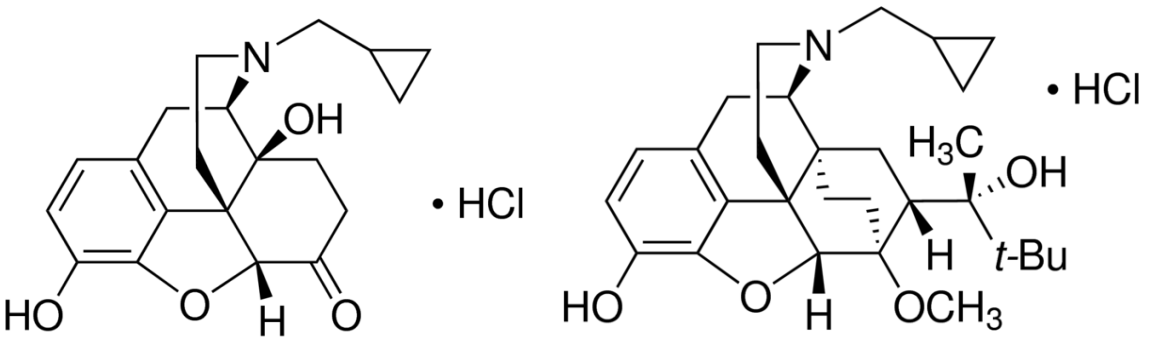

Figure 1.4: Structure of naltrexone hydrochloride (**left**) and buprenorphine hydrochloride (**right**).

Strategies explored by other groups include delivering prodrugs of NTX (Vaddi *et al.* 2009) and delivering the active metabolite of NTX (Paudel *et al.* 2005), and have had limited success. A strategy of compromising the barrier of the stratum corneum using microneedles (Wermeling *et al*. 2008; Ghosh *et al.* 2013) did result in fluxes higher than the target. However, it was considered that for this project, where chronic use of patches was anticipated, a microneedle system was not the most suitable option.

The fluxes of BUP (base) from marketed passive patches for analgesia are 0.8 - 1.4 $\mu g/cm^2/h$ (product information). This indicates that, unlike NTX, passive delivery rates might be sufficient to deliver BUP for relapse prevention. For completeness, table 1.3 shows some studies showing the passive flux of BUP measured *in vitro*.

Table 1.3: Previous *in vitro* studies on passive transdermal delivery of BUP.

Author	Approach	Details	Maximum flux achieved (μg/h/cm^2)
Roy *et al*. 1994	BUP as base or hydrochloride salt	Heat separated human epidermis, 50% ethanol in receiver solution	2.8 for the base and 8.2 for the salt, both from 90 % propylene glycol
Stinchcomb *et al*. 1996	BUP as base or prodrugs	Prodrugs, human skin	0.1 for BUP, all prodrugs lower
Wang *et al.* 2009	BUP as hydrochloride salt, lipid nanoparticles and prodrugs	Nude mouse skin, 50% ethanol in receiver solution	6.4 (BUP control) to 8.3[a] (best BUP nanoparticle formulation) Prodrugs all lower fluxes than BUP

Rationale for iontophoretic delivery of NTX and BUP

The literature indicates that NTX is not a strong candidate for passive transdermal delivery, and that predicted required fluxes might be difficult to achieve. The strategy selected here for optimising its flux was iontophoresis. Iontophoresis involves application of an electrical field across the skin at a low current density (up to 0.5 mA/cm^2). A transdermal iontophoretic device consists of two electrodes (drug is present at one, the other is a 'return' electrode) typically incorporated into a small patch containing microelectronics and a battery. Compared to passive delivery, iontophoresis can greatly increase the rate at which drugs can be delivered across the skin (Merino *et al.* 1997). It does so by applying a driving force to the drug molecule, and not by decreasing the barrier properties of the skin.

During iontophoresis, molecules move across the skin by three mechanisms: electrorepulsion (ER), electroosmosis (EO), and passive diffusion. Electrorepulsion drives ions into the skin away from the electrode of the like sign. For example, a cationic drug present at the anode can be transported into the body. Electrosmosis is a convective solvent flow that occurs when a current is passed across the skin. Electrorepulsion concerns only ionised molecules, whilst electrosmosis concerns both ionised and unionised molecules. Lastly, of course passive diffusion still occurs during iontophoresis, though obviously in a system optimised for iontophoretic delivery, the contribution of passive flux to total flux is likely to be negligible.

NTX has been previously identified as a good candidate for iontophoretic delivery (Giannola *et al.* 2007), due to its hydrophilicity and the fact that it is charged at a physiological pH (it is

a weak base with a pKa of ~8), however, in that work, the suggested site of iontophoresis was the buccal cavity.

As mentioned, it has already been demonstrated that BUP can be delivered transdermally by passive diffusion alone. However, in this thesis, BUP will be delivered by iontophoresis in order to be formulated together with NTX. It is important that BUP is physically incorporated into a product with NTX. As an opioid agonist, BUP is abusable in the absence of the antagonist NTX. Furthermore, transdermal delivery by iontophoresis offers increased control over input rate. Flux during iontophoresis is controlled by the current passed and not by the efficiency of the skin barrier (Harper Bellantone *et al.* 1986), consequently, variability (between body sites, and between individuals) is smaller than for passive transdermal delivery (Phipps *et al.* 1989). Iontophoresis also offers the possibility of faster attainment of therapeutic plasma levels compared to passive delivery (Thysman *et al.* 1994; Brand & Guy 1995; Singh *et al.* 1995). Lastly, unlike passive patches, iontophoresis offers the possibility of manipulating or titrating the dose during patch use. The main disadvantage of iontophoresis compared to the conventional oral route, and indeed compared to passive transdermal delivery, is likely to be an increased cost.

What type of molecule can be delivered by iontophoresis?

Clearly, for drugs to be delivered by electrorepulsion, they must be ionisable over the pH range of the skin (Kalia *et al.* 2004). In contrast, electroosmosis will act on all molecules, ionised or unionised.

It is easier for smaller molecules to cross the skin by electrorepulsion than larger molecules (Yoshida & Roberts 1992). However, the upper size limit of the permeant is significantly higher for iontophoresis than for passive, and peptides of a few kDa have been delivered (Green 1996).

A minimum aqueous solubility of 1 mg/ml is desirable to formulate for iontophoresis, and lipophilic molecules are not easily delivered (Prausnitz *et al.* 2004). In experiments performed using homologous series of compounds iontophoretic flux has been observed to decrease with increasing logP and molecular weight (Del Terzo *et al.* 1989; Lopez *et al.* 2003). Although in these studies it is not possible to dissociate effect of molecular weight from effect of logP, it is likely that effect of molecular size dominates (van der Geest *et al.* 1996; Mudry *et al.* 2007). Figure 1.5 shows the relationship between molecular weight and efficiency of iontophoretic delivery.

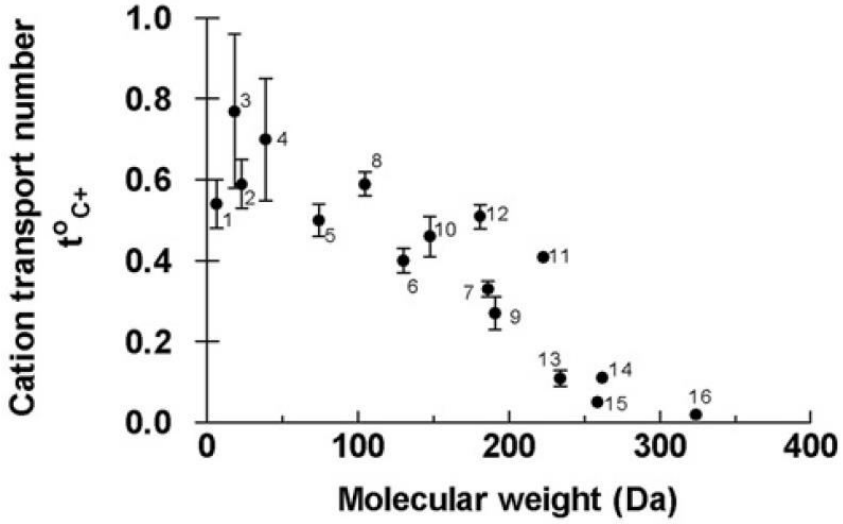

Figure 1.5: Cationic transport numbers in the single-carrier situation as a function of molecular weight. Transport number is simply the fraction of charge carried by the permeant and is therefore a measure of the efficiency of delivery. Taken from Mudry *et al.* (2007) and reproduced with kind permission from Elsevier.

Lastly, in contrast to passive delivery, it is likely that for iontophoresis the hair follicle route provides a major pathway for transport (Uitto & White 2003). That said, the contribution of this route during iontophoresis is probably system- and molecule-dependent, with preference for the follicle route increasing with hydrophilicity of the permeant (Cullander 1992).

Commercially available transdermal iontophoretic devices

Some iontophoretic devices have been approved for drug delivery. The Iomed Phoresor® system uses a small power pack in conjunction with disposable, fillable patches (provided without drug), and is commonly used to deliver dexamethasone to treat sports injuries. Prefilled patches are available for local delivery of lidocaine (for anaesthesia), for systemic delivery of fentanyl (for analgesia), and for systemic delivery of sumatriptan (for treatment of migraines). The lidocaine device is interesting as it also delivers epinephrine (a vasoconstrictor) to prolong local drug levels of lidocaine.

Table 1.4 shows the iontophoretic transdermal drug delivery products that have reached the market, and some properties of the drug molecules therein, alongside NTX and BUP for comparison. It can be seen that in terms of physicochemical properties, NTX and BUP are reasonable candidates for iontophoretic delivery.

Table 1.4: Selected physicochemical properties of molecules in marketed iontophoresis products, compared with properties of NTX and BUP. *Patch is provided without drug, dexamethasone is added to the patch at point of use.

		Molecular weight	logP	Water solubility (mg/ml)	Delivery electrode
Dexamethasone sodium phosphate*	Sports injuries, joint inflammation	392	1.83	50	Cathode
Fentanyl hydrochloride	Pain management	372	3.84	10	Anode
Lidocaine hydrochloride	Local anaesthesia	234	2.30	50	Anode
Sumatriptan succinate	Migraine	295	0.96	83	Anode
NTX hydrochloride	*Relapse prevention combination*	*378*	*1.7*	*60 (at pH 6)*	*Anode*
BUP hydrochloride		*504*	*2.63*	*1 (at pH 6)*	*Anode*

NTX is similar in size and lipophilicity to compounds demonstrated to be suitable for iontophoretic delivery. BUP is slightly larger and has a lower aqueous solubility.

Target iontophoretic transdermal fluxes for a BUP/NTX relapse prevention therapy

For the prediction of required passive fluxes, a patch size of 50 cm^2 was used. However, for an iontophoretic patch of 50 cm^2, the delivery electrode would occupy only half of that area (the other half would be the return electrode). The required iontophoretic fluxes are therefore calculated based on a 25 cm^2 delivery electrode area. The required iontophoretic flux of NTX is 15.2 $\mu g/cm^2/hr$, and the required iontophoretic flux of BUP is 2.2 $\mu g/cm^2/hr$.

No studies of transdermal iontophoresis of NTX have been published. The table below shows studies on transdermal iontophoresis of BUP. The authors of these studies did not discuss the advantages of iontophoretic delivery of BUP, but Banga (2011) suggests that transdermal iontophoresis of BUP (for analgesia) could offer higher plasma levels, better reproducibility, and rapid onset time, compared to passive delivery.

Table 1.5: Previous studies on iontophoretic transdermal delivery of BUP.

Author	Approach	Details	Maximum flux achieved ($\mu g/h/cm^2$)
Bose *et al*. 2001	Iontophoresis, BUP as hydrochloride salt	Human skin *in vitro*	< 1
Fang *et al*. 2002	Iontophoresis and electroporation, BUP as hydrochloride salt	Nude mouse skin *in vitro*	10.1 (iontophoresis) and 6.1 (electroporation)
Denuzzio *et al*. 1997	Iontophoresis, BUP as hydrochloride salt	*In vivo* in pig	Flux not given, 0.7 ng/ml C_{plasma} reached

Studying iontophoresis in vitro

For development and mechanistic studies, *in vitro* experiments are performed, commonly with human, pig or mouse skin (WHO 2006; Seto *et al.* 2010). The skin can be used 'as is' or sectioned to a uniform thickness with a dermatome (a surgical instrument that is used to cut skin for grafts). For a permeation experiment, the skin is mounted in a cell with the outer surface of the skin in contact with the drug (whether in a gel, cream, patch, or drug solution), and the inner surface of the skin in contact with a 'receiver' chamber. The receiver chamber is filled with buffer that can be sampled at intervals and assayed to quantify how much drug has crossed the skin in a given period of time.

When performing iontophoresis *in vitro,* 'side-bi-side' cells (see figure 1.6) are commonly used. Most often, cells with two chambers are used (the donor, which contains the drug, and the receiver); the return electrode sits in the receiver compartment. Both compartments can be magnetically stirred.

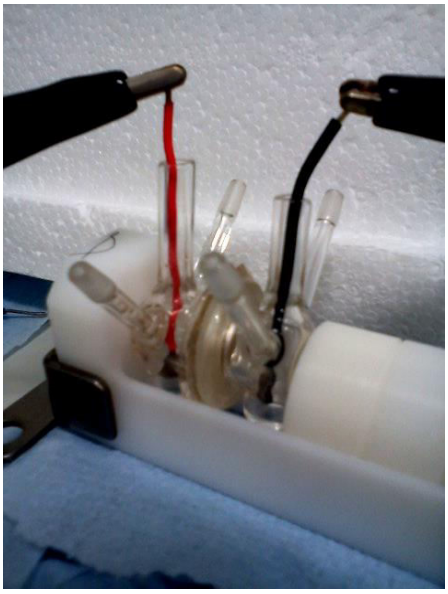

Figure 1.6: Photograph showing an example of an *in vitro* iontophoresis cell. Pig skin is clamped between the two glass compartments. Ag/AgCl electrodes are immersed in the fluid bathing the skin, without contacting the tissue. The side arms of the cell compartments can be used to withdraw samples from the receiver (or donor) during the experiment.

Following iontophoresis, the entire piece of skin can be assayed for drug; more precise information about the distribution of drug in the skin can be obtained by tape stripping. Tape stripping is a technique whereby successive layers of the stratum corneum are collected using adhesive tape, and from which drug can be extracted and assayed (Herkenne *et al*. 2006).

Overall, the background literature makes a compelling case for the combined iontophoretic delivery of BUP and NTX as a potential relapse prevention treatment to be explored.

3. **Aims and organisation of the thesis**

The first aim of the thesis was to assess the efficacy of a BUP/NTX combination to protect against relapse following a morphine or cocaine drug prime. In Chapter 2, the ratio of buprenorphine/naltrexone which was neither aversive nor rewarding was determined. Experiments were performed *in vivo*, using Sprague Dawley rats, with drug administered by intraperitoneal injection. The tail flick assay and the conditioned place preference method were used. In Chapter 3, it was demonstrated that buprenorphine/naltrexone may be effective at reducing both morphine- and cocaine-seeking. This was achieved using a conditioned place preference method which was extended to include an extinction and reinstatement component.

The second aim of the thesis was to assess the transdermal route as an administration option for BUP and NTX together. Chapters 4 and 5 concern the co-formulation of buprenorphine and naltrexone for delivery by transdermal iontophoresis. Experiments were performed *in vitro*, using pig skin. Chapter 4 demonstrated that these two drugs can be transported across the skin by iontophoresis, and showed the effect of pH, drug concentration, and current density. Chapter 5 characterised the effect of receiver solution and current profile.

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Chapter 2: Establishing a non-aversive, non-rewarding ratio of naltrexone:buprenorphine

1. Introduction

An important feature of a buprenorphine/naltrexone (BUP/NTX) combination is that it should be neither rewarding nor aversive. Too much BUP could lead to a therapy that is rewarding, via agonism of the mu receptor (Marquez *et al*. 2007). This might be unacceptable from a regulatory point of view, and would be inappropriate for use in opioid-naïve cocaine-dependent patients. Too much NTX could lead to a therapy that is aversive, possibly via mu antagonism effects on mood (Parker & Rennie 1992; Suzuki *et al*. 1992; Miotto *et al*. 2002); this would probably result in low retention rates (compliance currently being a major problem with NTX alone). The aim of this chapter was, therefore, to determine the ratio of BUP/NTX that was neither rewarding nor aversive.

In the small clinical studies carried out by Rothman *et al*. (2000) and Gerra *et al*. (2006), NTX and BUP were administered at a ratio of approximately 10:1. Based on observations of the pupil diameters of patients, it was reported that at this ratio (and dose), the combination produced no overall mu agonism. It is possible that a ratio of 10:1 represents a cautious approach, and that more mu agonism could be tolerated without resulting in rewarding effects. Further, a low level of mu agonism might be desirable for improved compliance (Nutt 2010).

In man, information about levels of mu agonism produced by different doses or different ratios of drugs can be relatively easily obtained by self-reporting; study participants are administered the drug/s then asked questions such as “How high are you?” or “Do you feel any drug effect?” (Schuh *et al.* 1999). In the present study, experiments to determine the mu agonism produced by various combinations of NTX:BUP were performed in rats. In animal models, reward produced by a drug or combinations of drugs can be studied using self-administration studies or conditioned place preference (CPP). Alternatively, the amount of mu agonism produced by a drug or combinations of drugs can be indirectly determined by measuring analgesia or mu antagonist-precipitated withdrawal.

In an assay of analgesia, the animal is exposed to a pain-producing stimulus (such as heat or mild electric shock), and the response is measured (such as time taken to react to the heat stimulus, or the magnitude of electric shock which elicits vocalization). The mu antagonist-precipitated withdrawal assay takes advantage of the fact that if a drug produces significant mu agonism, then subsequent administration of a sufficient dose of mu antagonist

will produce signs of withdrawal such as diarrhoea and teeth-chattering that can be scored by the experimenter; greater withdrawal indicates greater prior mu agonism (Wee *et al.* 2012). In self-administration studies, rodents or non-human primates can be trained to press a lever to receive a dose of a mu agonist, typically via an intravenous catheter (Shalev *et al*. 2002). Lastly, CPP is a behavioural method commonly used in rodents for measuring drug-induced reward (Cunningham *et al*. 2006; Tzschentke 2007). The animal is conditioned to associate a reward (the subjective effects of a drug) with a particular compartment. The compartments will be referred to as the drug-paired (DP) compartment and the saline-paired (SP) compartment. The strength of conditioning can be quantified by measuring the amount of time spent in the drug-paired compartment when the animal is allowed to choose between compartments in a drug-free state.

It is generally accepted that whilst both self-administration and CPP have good predictive validity of whether a substance will be abused in man, they measure slightly different things. CPP is thought to measure the rewarding (or aversive) motivational properties of a drug (Cunningham *et al.* 2006, Tschentke 2007). Self-administration measures how reinforcing a drug is, and how hard an animal will work to receive the drug (Epstein *et al*. 2006).

In this chapter, analgesia was selected to measure mu agonism, and CPP was selected to measure drug reward, following administration of various ratios of NTX:BUP. The specific analgesia assay chosen was the hot water tail withdrawal assay. The tail of the rat was placed in hot water and the time taken for the tail to be withdrawn was recorded. Any analgesic effect observed was attributed to mu agonism by BUP (Dum & Herz 1981; Lutfy *et al.* 2003). The CPP method has been used successfully in the past to measure the rewarding properties of differing doses (Bardo *et al*. 1995) and combinations (Canestrelli *et al*. 2014) of drugs, including mu agonists. CPP is a good predictor of rewarding properties/abusability in man (Bardo *et al.* 1995). Lastly, the CPP method also allows measurement of aversion (Cunningham *et al.* 2006). If the compound tested is aversive, the animal will spend *less* time in the drug-paired compartment than at baseline; this is referred to as conditioned place aversion.

Rodent studies in which indicators of mu agonism were measured following co-administration of BUP and NTX are shown in table 2.1. Overall, these studies indicate that ratios of NTX:BUP less than 10:1 are not necessarily rewarding. The ratios of NTX:BUP selected for testing in this chapter were therefore 10:1, 3:1 and 1:1.

Table 2.1: Rodent studies in the literature that measured indicators of mu agonism produced following co-administration of BUP and NTX.
*A discrimination assay is generally performed using a two-lever operant procedure (Huskinson *et al.* 2014). The animal is trained to press lever A when it receives an infusion of the training drug (delivered by the experimenter) and to press lever B when it is receives an infusion of saline (delivered by the experimenter). This training is accomplished by reward of correct responses with food, for example. Once training is complete, a test drug can be administered. Pressing of lever A would then indicate that the test drug has a similar subjective effect to the training drug.

.	Study details	NTX:BUP ratio	Outcome
Dum & Herz 1981	Rats, analgesia	5:1	mu agonism still evident, NTX administered first
Walker *et al*. 1994	Rats, analgesia	3:1	no mu agonism observed
Walker *et al*. 1994	Rats, discrimination*	3:1	no mu agonism observed
Holtzman 1997	Rats, discrimination*	3:1	no mu agonism observed
Kögel *et al.* 2005	Mice, analgesia	3:2	mu agonism blocked if NTX administered first, or unchanged if BUP administered first
Wee *et al.* 2012	Rats, analgesia	1:10	mu agonism still evident
Wee *et al.* 2012	Rats, mu antagonist-precipitated withdrawal	1:10	mu agonism still evident
Almatroudi *et al*. 2015	Mice, analgesia	1:1	no mu agonism observed

2. Materials and methods

Drugs and chemicals

Naltrexone hydrochloride dihydrate was from Fluka (UK). Buprenorphine hydrochloride was prepared in-house. Saline (sodium chloride 0.9%) was from Dechra (UK). All injections were intraperitoneal (1 ml/kg).

Animals

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and the University of Bath's ethical review documents. Male Sprague Dawley rats (Charles River, UK) were used; 260-420g (7-11 weeks old) for tail withdrawal, 235-345g (7-9 weeks old) for conditioned place preference experiments. All rats were housed four per cage with *ad libitum* access to food and water and maintained on a 12:12 h light-dark cycle (lights on 07:00, lights off 19:00).

Tail withdrawal assay

A water bath (Grant Instruments, UK) was maintained at a constant temperature. 52ºC was selected, after demonstrating that at 55ºC 0.3 mg/kg BUP did not elicit any measurable analgesia. The rats were held firmly in a vertical position, and lowered until the distal third of the tail was in the water. The time taken for the rat to withdraw the tail was recorded. A 20 second cut-off was imposed to avoid tissue damage. All rats were opioid naïve, and were not reused. A saline-only control was included.

Conditioned place preference apparatus

CPP boxes (Tracksys, UK) were three-chambered shuttle boxes comprising a small central compartment (10 x 10 cm) where rats were placed at the start of a test session, and two larger compartments (40 x 40 cm), one with horizontal black and white stripes, and one with vertical black and white stripes. Floors were made of stainless steel sheeting with punched-out shapes (circles, 12 mm hole, and squares, 10 mm hole) resulting in distinct textures (Novametals, UK). Removable partitions allowed the boxes to be used either to restrict the rats to a particular compartment for conditioning, or to allow the rats to be 'free-to-explore' during a test session. Experiments were performed between 8 am and 5 pm under dim white light (light intensity approximately 15 lux). During all test sessions, the time each rat spent in each compartment was recorded using EthoVision XT (Tracksys, UK) tracking software.

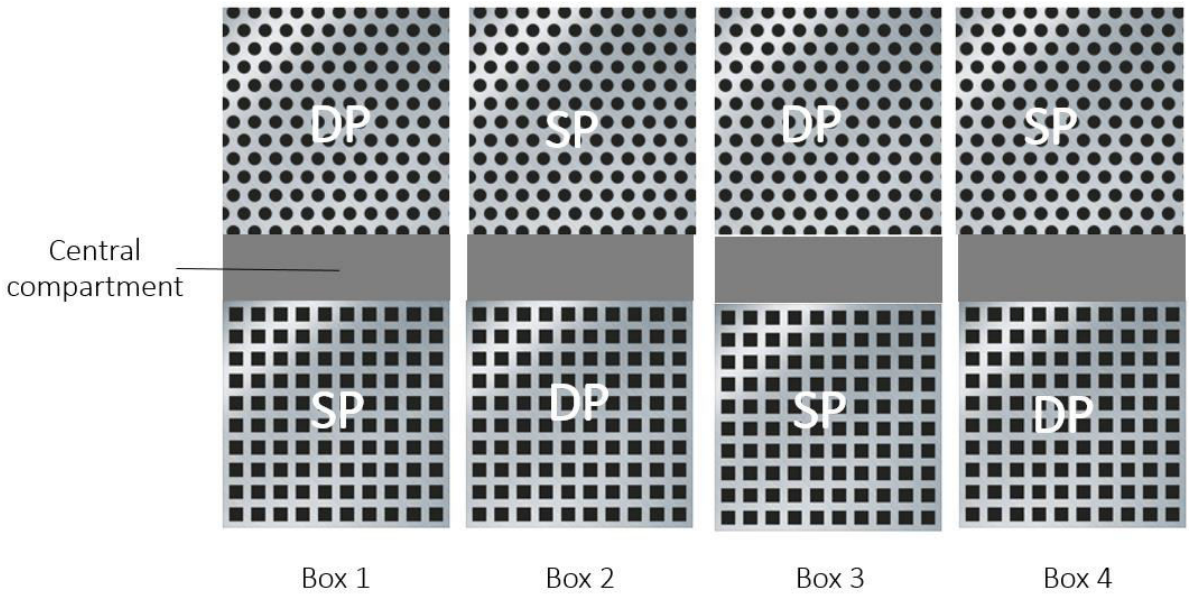

Figure 2.1: Layout of CPP boxes. DP = drug-paired compartment. SP = saline paired-compartment. The flooring of the CPP apparatus was selected with the aim of the two compartments being distinct enough for the rats to be able to acquire a place preference, but equivalent enough that no innate preference for either compartment would be observed. One rat was placed in each CPP box; four tests were run simultaneously.

Within each cohort, half the rats were trained to associate the compartment with the circle flooring with a drug reward, and half the rats were trained to associate the compartment with the square flooring with a drug reward (figure 2.1).

The CPP data throughout are presented after multiplying by a correction factor (following Brenhouse & Andersen 2008). The correction factor is useful when the CPP apparatus includes a neutral central compartment, because a change in the time that a rat spends in the drug-paired compartment does not necessarily mean that a change in preference has occurred. This is illustrated in table 2.2. If only the uncorrected time spent in the drug-paired compartment is examined it appears that the preference of the rat for the drug-paired compartment is very different across the three sessions. In actual fact, in this example, the preference of the rat for the drug-paired compartment over the saline-paired compartment is identical in all three sessions, it is only the time spent in the central compartment that varies.

Table 2.2: An illustration of the correction factor employed to remove any influence of time spent in the central compartment on the apparent preference of a rat for the drug-paired compartment over the saline-paired compartment.

	Time in drug-paired compartment (s)	Time in central compartment (s)	Time in saline-paired compartment (s)	Corrected time in drug-paired compartment (s)
Test session A	400	300	200	600
Test session B	600	0	300	600
Test session C	200	600	100	600

Equation 2.1 shows how the corrected value was calculated. Effectively, the time spent in the neutral central compartment is divided proportionally between the two conditioning compartments (Brenhouse & Andersen 2008).

Equation 2.1

$$\text{Corrected time in DP compartment (s)} = \left(\frac{900}{\text{actual time in DP + SP compartment (s)}}\right) \text{x actual time in DP compartment (s)}$$

A value of 900 is used in this equation (and in equation 2.2) because there are 900 seconds in a 15 minute test period.

The corrected values of time spent in the drug-paired compartment were used to generate a preference score (equation 2.2). If a rat spent zero time in the saline-paired compartment during a test session the preference score was 50. If a rat spent zero time in the drug-paired compartment the preference score was -50.

Equation 2.2

$$\text{Preference score} = \left(\frac{\text{Corrected time in DP compartment (s)} - 450}{900}\right) \text{x } 100$$

Individual rats which spent more than 600 or less than 300 seconds in the drug-paired compartment (equivalent to a preference score of >16.7 or < -16.7) during the baseline session were excluded (following Ribeiro Do Couto *et al*. 2006). Groups were organised such that mean baseline preference scores were close to zero.

Assessment of the mu agonism/antagonism of the NTX/BUP combination

In these experiments, the amount of BUP administered was fixed at 0.3 mg/kg per dose (after Tzschentke 2004). The same BUP dose was administered alone to serve as a positive control, and it was expected that BUP's mu agonism effects would be observable at this dose in both the tail withdrawal assay (Walker *et al.* 1994) and in the CPP method (Rowlett *et al.* 1994). BUP was also administered (in separate cohorts) in combination with NTX. For the tail withdrawal assay, doses of NTX were either 0.3 or 1 mg/kg, resulting in approximate ratios of NTX:BUP of 1:1 or 3:1. For the CPP method, doses of NTX were either 0.3, 1 or 3 mg/kg, resulting in approximate ratios of NTX:BUP of 1:1, 3:1 or 10:1. Lastly, for the CPP method, one group of rats was administered 3.0 mg/kg NTX only.

Tail withdrawal assay. Five baseline measurements were taken, one immediately after another, for each rat. Baseline tail-withdrawal time was taken as the mean of the last 2 baseline measurements. 0.3 mg/kg BUP was then administered in combination with either 0, 0.3 or 1.0 mg/kg NTX (n = 5, 4, 7). Following injection of the drugs, measurements (as described previously) were taken once every 7.5 minutes, up to 60 minutes; data collected at the 60 minute time-point was used in subsequent analyses. For the saline control group (n = 4), measurements were taken up to 30 minutes. For each rat, analgesia was quantified as Δ_t (increase in tail-withdrawal time compared to baseline measurement). The Δ_t group means were compared using one-way ANOVA with the Bonferroni post-test.

CPP assay. Rats were conditioned with BUP (0.3 mg/kg) administered in combination with either 0, 0.3, 1 or 3 mg/kg NTX (n = 16, 8, 8, 16). One group of rats were conditioned with 3.0 mg/kg NTX only (n = 8). Figure 2.2 shows the time course of a typical experiment.

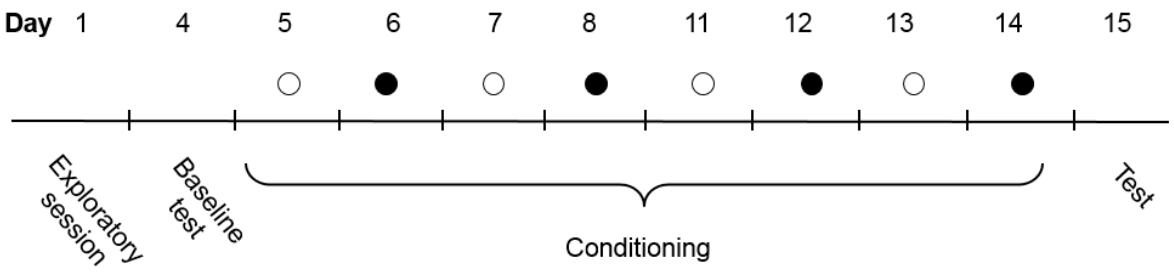

Figure 2.2: Schematic of an example time course of the conditioned place preference method. Filled-in circles represent drug injections and empty circles represent saline injections.

On day 1, rats had a 15 minute exploratory session; on day 4, they had a 15 minute baseline preference test. On days 5-8 and 11-14, the rats received drug or saline on alternate days, thus, each rat had 4 drug injections and 4 saline injections. Injections were

administered at least 24 hours apart to ensure that the effects of BUP had dissipated before subsequent saline injections (Tzschentke 2004). Following injections, the rats were immediately confined to a compartment for 40 minutes. On day 15, % preference was obtained exactly as for baseline preference; i.e., the preference for each drug treatment was measured by recording the time spent in the drug-paired chamber in a free-to-explore test lasting 15 minutes. To assess conditioning, a 1-tailed Wilcoxon matched pairs signed-rank test was used (each group's preference score after drug treatment compared to its baseline).

In an optimal study design, only groups of equal sample size (n numbers) should be compared, to avoid confounding. However, throughout this thesis groups of unequal sizes were used (for example in figure 2.3 and table 2.3). This represents a compromise due to time and cost factors.

3. Results and Discussion

The aim of this chapter was to find out the minimum amount of NTX required to block the rewarding effects of BUP. Two separate assays were selected; the first was a measurement of analgesia and therefore an indirect measure of mu agonism, and the second was a behavioural assay and a direct measure of drug reward.

Using NTX to block the rewarding effects of BUP

Tail withdrawal assay. Table 2.3 and figure 2.3 show that, as expected, when administered alone, 0.3 mg/kg BUP elicited marked analgesia. This can be seen by the increase in time taken to withdraw the tail compared to baseline (Δ_t). The full analgesic effect from BUP emerged after approximately 20 minutes. This analgesic effect was blocked by the inclusion of 1.0 mg/kg NTX; Δ_t at 60 minutes was statistically different from the BUP only group (one-way ANOVA with the Bonferroni post-test, $p < 0.05$). Inclusion of 0.3 mg/kg NTX resulted in a Δ_t that was intermediate between that observed for 0 and 1.0 mg/kg NTX, and was not statistically different from either group.

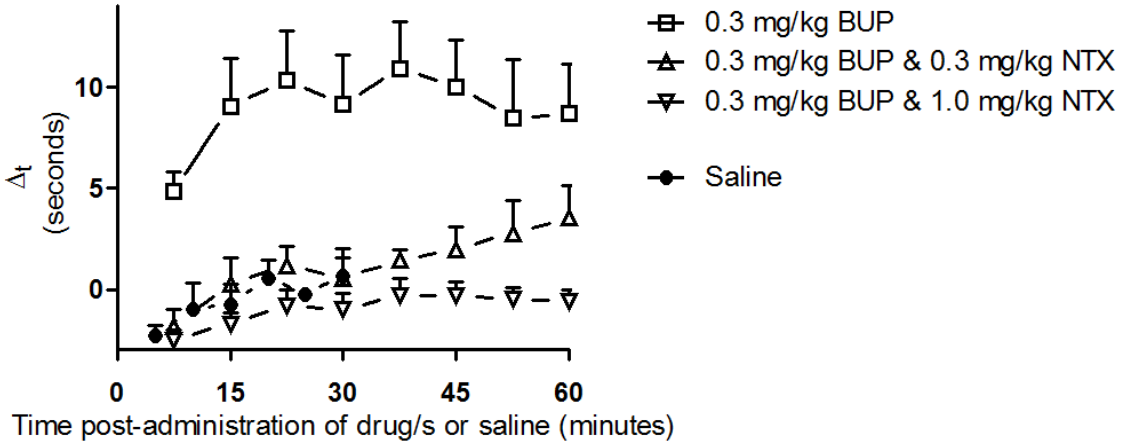

Figure 2.3: Analgesic effects of 0.3 mg/kg BUP administered in combination with 0, 0.3 or 1.0 mg/kg NTX (n = 5, 4 and 7 respectively). Time (mean ± SEM) taken for rats to withdraw tail during the hot water assay was measured. A saline control was also performed (n = 4). The 60 minute Δ_t values were compared (one-way ANOVA with the Bonferroni post-test) and the 0.3 mg/kg BUP group was significantly different ($p < 0.05$) from the 0.3 mg/kg BUP & 1.0 mg/kg NTX group.

Table 2.3: Analgesic effects of 0.3 mg/kg BUP administered in combination with 0, 0.3 or 1.0 mg/kg NTX (n = 5, 4 and 7 respectively). Time (mean ± SEM) taken for rats to withdraw tail during the hot water assay was measured. Withdrawal times shown are measured 60 minutes after administration of drug/s.

BUP & NTX (mg/kg)	0.3 & 0	0.3 & 0.3	0.3 & 1.0
Withdrawal time at baseline (s)	4.0 ± 0.4	5.5 ± 0.6	5.2 ± 0.6
Withdrawal time after administration of BUP/NTX (s)	12.6 ± 2.1	9.0 ± 1.7	4.6 ± 0.3
Δ_t (s)	8.7 ± 2.2	3.5 ± 1.6	-0.6 ± 0.6

The results from this analgesia assay indicated that 1.0 mg/kg of NTX could block the mu agonism of 0.3 mg/kg BUP. This was then tested in the CPP assay, which is used to measure drug reward.

CPP assay. One rat in the 0.3 mg/kg BUP group, one rat in the 0.3 mg/kg BUP with 0.3 mg/kg NTX group, and one rat in the 3.0 mg/kg NTX only group were excluded for having a preference at baseline. The results of the baseline test are shown in figure 2.4, and the results of the post-conditioning test are shown in figure 2.5; table 2.4 summarises the

results. As expected, 0.3 mg/kg BUP alone elicited drug-seeking behaviour in the CPP method. This can be seen as a significant increase in the time spent in the drug-paired compartment by that group compared to its baseline (1-tailed Wilcoxon matched pairs signed-rank test, $p < 0.05$).

Table 2.4: Rewarding effects of 0.3 mg/kg BUP administered in combination with 0, 0.3, 1.0 or 3.0 mg/kg NTX (n = 15, 7, 8, 16 respectively), and 3.0 mg/kg NTX alone (n = 7). Time (mean ± SEM) spent in the drug-paired compartment at baseline, and after conditioning. The test session was 15 minutes, so a score of 450 indicates equal time spent in each compartment.

BUP & NTX (mg/kg)	0.3 & 0	0.3 & 0.3	0.3 & 1.0	0.3 & 3.0	0 & 3.0
Baseline (s)	447 ± 16	465 ± 41	465 ± 18	448 ± 15	418 ± 28
After conditioning (s)	544 ± 34	508 ± 62	436 ± 60	355 ± 42	455 ± 23
After conditioning (preference score)	10.4 ± 3.7	6.5 ± 6.9	-1.5 ± 6.6	-10.6 ± 4.6	0.6 ± 2.6

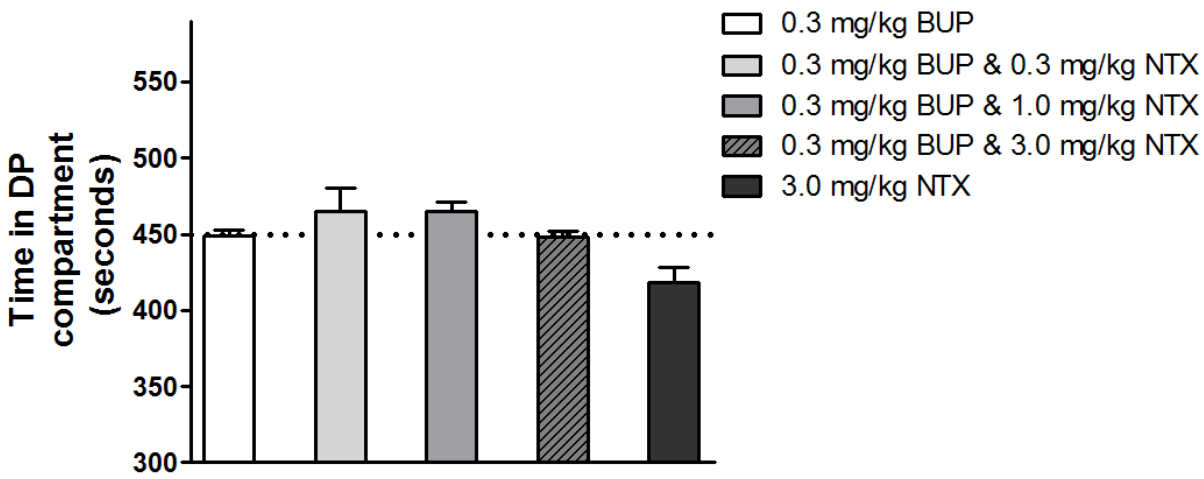

Figure 2.4: Time spent in the drug-paired compartment during the baseline test (mean + SEM of n = 15, 7, 8, 16, 7). The dotted line at 450 indicates equal time spent in both compartments; test duration being 15 minutes.

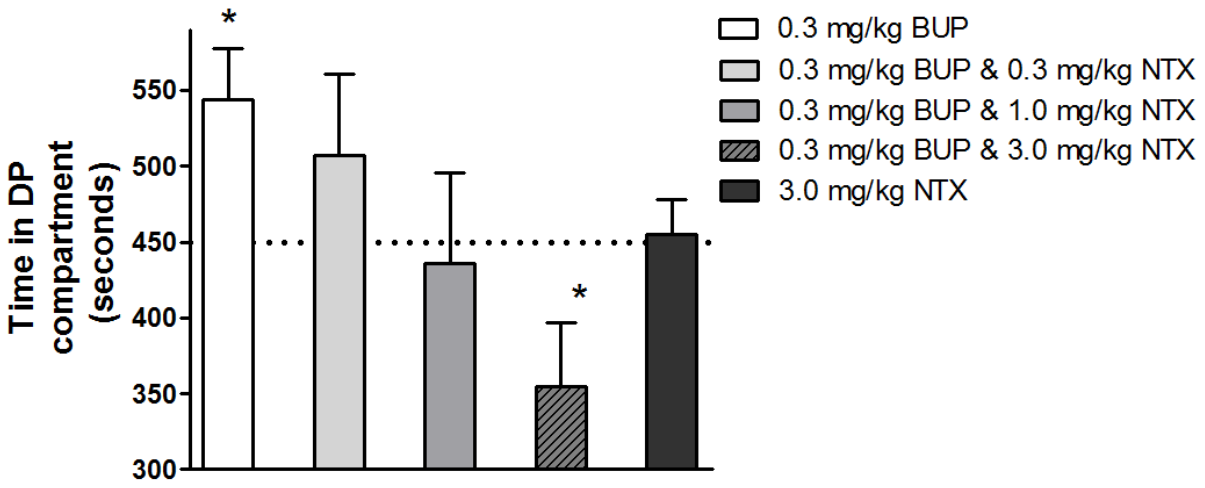

Figure 2.5: Time spent in the drug-paired compartment after conditioning (mean + SEM of n = 15, 7, 8, 16, 7). * indicates preference score was significantly different from baseline (1-tailed Wilcoxon matched pairs signed-rank test, $p < 0.05$). The dotted line at 450 indicates equal time spent in both compartments; test duration being 15 minutes.

For the groups which received 0.3 or 1.0 mg/kg NTX, time spent in the drug-paired compartment was not significantly different from their respective baselines. The group which received 0.3 mg/kg BUP with 1.0 mg/kg NTX showed very little change from its baseline, indicating that this dose combination was neither rewarding nor aversive.

Interestingly, NTX countered the rewarding effects of BUP to the extent that co-administration of 0.3 mg/kg BUP with 3.0 mg/kg NTX actually elicited aversion (rats spent significantly more time in the saline–paired compartment compared to baseline test). Previous studies have indicated that mu antagonists can be aversive. Mucha *et al.* (1985) reported observing a conditioned place aversion in rats following administration of naloxone. Hollister *et al.* (1981) reported that NTX caused “intolerably unpleasant” effects including “loss of energy, gastrointestinal disturbances and mental depression” in healthy volunteers with no history of illicit drug use. The apparent aversive effects of mu antagonists has been attributed to blockade of endogenous opioid activity (Colasanti *et al*. 2011).

To determine if the aversion observed after administration of 0.3 mg/kg BUP with 3.0 mg/kg NTX was due to mu antagonism by NTX, a conditioned place preference experiment was carried out in which only 3.0 mg/kg NTX was administered. Unexpectedly, no aversion was observed when NTX was administered alone, and the time spent in the drug-paired compartment was not different from baseline. A similar result has been seen by Canestrelli *et al.* (2014) who used CPP to assess the effect of administering BUP in combination with the mu antagonist, naloxone. They observed that combinations of BUP and naloxone could

be aversive, whereas the same dose of naloxone on its own was not. Canestrelli's hypothesis was that naloxone masked the mu agonism of BUP, allowing the actions of BUP on another receptor, NOP, to be observed. Indeed, they showed that administration of an NOP antagonist blocked the aversive properties of the BUP/naloxone combination. Our data seem to support their hypothesis. Intriguingly, there is a growing body of evidence that NOP has anti-addictive actions (Kotlinska *et al.* 2002; Sakoori & Murphy 2004), though no mechanism has been demonstrated to date.

It is not known to what extent the data reported here, collected in rats, will translate to humans. As mentioned in the introduction, CPP models have good predictive validity for which substances will be rewarding in man, but in terms of dose and ratio, there are likely to be some pharmacokinetic and pharmacological interspecies differences.

The clearance of BUP and NTX in the rat is 1.41 and 4.02 L/kg/h respectively (Ohtani *et al.* 1995 & 1997; Akala *et al.* 2008). The clearance of BUP and NTX in humans assuming a 60 kg body weight is 1.28 and 3.16 L/kg/h respectively (Bullingham *et al.* 1980; Kuhlman *et al.* 1996; Lictko 1981; Wall *et al.* 1981 &1984). Although the reported clearances in rat are slightly higher than those for humans, the relative proportions of the clearance values for the two drugs are very similar. Yassen *et al.* (2007) described rate constants of BUP for association and dissociation at the mu receptor as identical in rat and man.

Lastly, there are metabolic differences between the two species; in contrast to man, significant levels of the metabolites norbuprenorphine or 6-β-naltrexol are not observed in rats (Ohtani *et al.* 1997; Akala *et al*. 2008). Overall, clearly the findings of this chapter would need to be confirmed in man.

4. Conclusions of the chapter

There was good agreement between the findings of the tail withdrawal assay and the CPP assay. Taken together, it was clear that, in these rats, when administered via the intraperitoneal route, 1.0 mg/kg NTX blocked the mu-opioid receptor agonism of 0.3 mg/kg BUP (a ratio of 3:1 NTX:BUP). Additionally, the CPP assay demonstrated that this combination is non-rewarding and non-aversive.

In the previous studies in man (Rothman *et al*. 2000; Gerra *et al*. 2006), NTX and BUP were administered at a ratio of approximately 10:1. Our finding hints that this ratio may represent an overly cautious approach, and that patients may be able to receive less NTX and still

have adequate blockade of BUP's rewarding effects. Furthermore, our finding suggests that in this combination, if too much NTX is administered, the combination may be aversive. This would need to be considered before new trials in man were planned.

In this work, the combination was administered by intraperitoneal injection whereas our long term aim was to deliver these compounds by the transdermal route. As stated previously, a critical feature for a potential anti-addiction therapy is that the treatment itself is not rewarding (Manlandro 2007). Subjective positive effects are linked with speed of onset (Samaha & Robinson 2005), and if no rewarding effects were observed when the combination was administered via the intraperitoneal route, it is unlikely that any rewarding effects would be present if the drug was delivered as a continuous input (as would be the case from a transdermal patch).

Lastly, the finding that 0.3 mg/kg BUP with 1.0 mg/kg NTX is not rewarding means that in chapter 3, when the ability of BUP/NTX to block relapse is tested using the conditioned place preference reinstatement model, we can expect that the BUP/NTX treatment itself will not act as a priming dose and trigger relapse.

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Chapter 3. Blocking reinstatement to drug-seeking behaviour with buprenorphine and naltrexone

1. Introduction

Relapse (resumption of drug use after a period of abstinence) can be triggered by stress, by cues that have become associated with drug-taking, or by a single, small dose of drug (Shalev *et al.* 2002; Aguilar *et al.* 2009). Current therapies protect against relapse to varying extents. For example, if NTX treatment is adhered to it will provide pharmacological blockade against heroin therefore will reduce the likelihood of a single drug use triggering a relapse (Schuh *et al*. 1999). Arguably the substitution therapies BUP and methadone reduce the likelihood of relapse by reducing the need to use heroin (Gerra *et al*. 2009). However, there is no evidence that any of these therapies are protective against relapse in the event of cocaine use.

The Rothman (2000) and Gerra (2006) clinical studies showed that a BUP/NTX combination is a potential relapse prevention therapy against both heroin and cocaine use. However, those studies were conducted using a ratio of 10:1 NTX:BUP, whereas the results in chapter 2 showed that (in rats at least) a ratio of 3:1 NTX:BUP was optimal in terms of the therapy itself being neither rewarding nor aversive. The first aim of this chapter was, therefore, to determine whether this smaller ratio of 3:1 NTX:BUP retained enough mu antagonist activity to block relapse following a morphine prime. Morphine was used here in place of heroin because heroin is quickly deacetylated to morphine in the body and in solution (Jones *et al*. 2013).

It is not possible to tell from the Rothman (2000) and Gerra (2006) studies whether the observed reduction in cocaine use was a direct effect of the BUP/NTX therapy on cocaine use, or a consequence of reduced heroin use. The pharmacological interaction between the opioids BUP/NTX and heroin is reasonably well understood, but a mechanism for the effect of BUP/NTX on cocaine use is not as clear. There is only one study (to the author's knowledge) that attempts to assess directly the effect of co-administered BUP/NTX on cocaine-seeking. Wee *et al.* (2012) trained rats to self-administer cocaine, then administered BUP, alone and with increasing amounts of NTX; their rationale for inclusion of NTX in the therapy was similar to ours. They observed that administration of BUP alone reduced self-administration for cocaine, but that increasing amounts of NTX reversed the effect of BUP. They concluded that as long as the amount of NTX in the combination was kept low, this combination reduced cocaine-seeking. However, these results could be explained equally well by the rewarding properties of BUP. The doses of BUP used in the

Wee study (2012) were relatively high (3 mg/kg for two consecutive days) and this dose has been shown to be rewarding (Walker *et al.* 1994, Rowlett *et al.* 1994). Therefore perhaps when the rat was administered BUP, it was satiated and did not need to self-administer cocaine. This explanation would fit with the observation that increasing doses of NTX cancelled BUP's effect on self-administration of cocaine. Overall, the effect of a BUP/NTX combination on cocaine-seeking is not clear, and therefore the second aim of this chapter was to measure the effect of BUP/NTX on drug-primed reinstatement to cocaine-seeking.

In this chapter, the CPP (conditioned place preference) method was used to measure the effect of the BUP/NTX treatment on cocaine-seeking and morphine-seeking in rats (separate cohorts). There are (at least) two different ways the CPP method can be used to measure the efficacy of anti-addiction therapies. The simplest is to measure if prior administration of the therapy can block acquisition of CPP to rewarding drugs (Sakoori & Murphy 2004). This approach can provide useful information, but obviously does not have strong construct validity. Therefore we chose the CPP-extinction-reinstatement method which we believe has more relevance to relapse prevention in an abstinent drug-dependent patient. Firstly, rats are conditioned to associate a particular compartment of the CPP apparatus with a drug reward. After that, the drug-seeking behaviour is extinguished ('extinction' is said to have occurred). Extinction is the loss of the drug-seeking behaviour, and occurs when the reward is no longer presented; the rats learn to stop a behaviour that has ceased to be productive, and drug-seeking falls to baseline levels (Rutten *et al.* 2011b; McNally 2014). The last stage of the model is reinstatement, the animal model equivalent of relapse. Reinstatement is simply resumption of drug-seeking after a period of extinction and occurs if the animal is exposed to a trigger such as stress or a single, small dose of drug. The CPP-extinction-reinstatement paradigm is frequently used to assess the efficacy of potential relapse prevention therapies (Aguilar *et al*. 2009).

In this work, reinstatement triggered by a drug prime was investigated. The term 'drug prime' is generally understood to mean a lower dose than that used for conditioning, and is commonly (but not always) the same drug as that used for conditioning. Drug-primed reinstatement is thought to occur because it gives a reminder of the subjective effects of the rewarding drug (Shalev *et al*. 2002). Drug-primed reinstatement was chosen for investigation because whilst there is a decent body of literature suggesting that kappa antagonists can block stress-triggered reinstatement to cocaine (reviewed by Bruchas *et al*. 2010), less is known about the effect of kappa antagonism on drug-primed reinstatement to cocaine. As well as their effects at the mu receptor, both BUP and NTX are kappa antagonists.

The CPP-extinction-reinstatement paradigm is relatively time-consuming (for example, one data set can take up to 3 weeks), so it was prudent to find another means of checking whether the proposed doses/ratio of BUP/NTX would be likely to block a priming dose of morphine before commencing behavioural work. Therefore, the tail withdrawal assay (which is relatively quick and simple compared to CPP) was used to measure the ability of 0.3 mg BUP with 1.0 mg/kg NTX to block 10 mg/kg morphine (which was anticipated to be larger than the priming dose used in the behavioural work). If no analgesia was observed, it would indicate that the BUP/NTX combination blocked the mu agonism of the morphine; this could then be tested in the CPP method.

Additionally, in order to know the plasma and brain concentrations of BUP and NTX in the rats at the time that the drug-prime was administered, a separate cohort of rats were administered the combination, and samples were collected and assayed. Lastly, receptor occupancies of BUP and NTX at the mu receptor and kappa receptor were predicted from the observed plasma and brain concentrations.

2. Materials and methods

Drugs and chemicals

Naltrexone hydrochloride dihydrate was from Fluka (UK). Buprenorphine hydrochloride was prepared in-house. Cocaine hydrochloride and morphine sulphate were from MacFarlan Smith (UK). Saline (sodium chloride 0.9%) was from Dechra (UK). Buffer components and mobile phase components were from Sigma (UK). All injections were intraperitoneal (1 ml/kg).

Animals

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and the University of Bath's ethical review documents. Male Sprague Dawley rats (Charles River, UK) were used; 260-420g (7-11 weeks old) for tail withdrawal, 250-320g (7-9 weeks old) for conditioned place preference experiments. All rats were housed four per cage with *ad libitum* access to food and water and maintained on a 12:12 h light-dark cycle (lights on 07:00, lights off 19:00).

Tail withdrawal assay

The tail withdrawal assay is described in the previous chapter; see page 40.

Conditioned place preference apparatus

The conditioned place preference assay is described in the previous chapter; see pages 40-42.

Measuring the antagonism of morphine by the BUP/NTX combination

Before behavioural work was undertaken, the tail withdrawal assay was used to establish whether the BUP/NTX combination could block the mu agonism of morphine. Five baseline measurements were taken, one immediately after another, for each rat. Baseline tail-withdrawal time was taken as the mean of the last 2 baseline measurements. Latency to withdrawal was measured following administration of 10 mg/kg morphine only (n = 4), and when both 0.3 mg/kg BUP and 1.0 mg/kg NTX (n = 5) or 1.0 mg/kg NTX alone (n = 7) were administered 30 minutes prior to the morphine. Following injection of the morphine, measurements were taken once every 5 minutes, up to 30 minutes. Data collected at the 30 minute time point was used in subsequent analyses. For each rat, analgesia was quantified as Δ_t (increase in tail-withdrawal time compared to baseline measurement). The Δ_t group means were compared using one-way ANOVA with the Bonferroni post-test.

Effects of BUP/NTX on reinstatement of cocaine and morphine conditioned place preference

To test the ability of a BUP/NTX combination to block drug-primed reinstatement, a CPP-extinction-reinstatement method was established (figure 3.1).

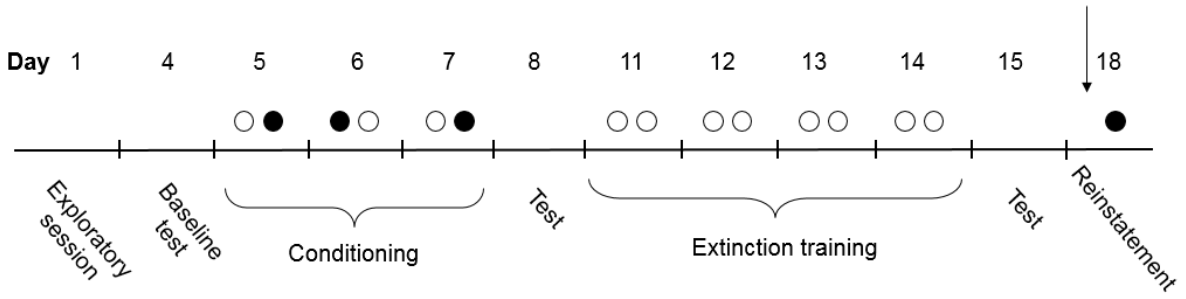

Figure 3.1: Schematic of the time course of the conditioned place preference extinction and reinstatement method. Filled-in circles represent cocaine or morphine injections and empty circles represent saline injections. During conditioning the order of drug and saline injections alternated daily. This schematic shows the reconditioning style of extinction, where each rat received 2 saline injections per day. The arrow indicates that BUP/NTX treatment (or saline, in the control groups) was administered 10 minutes prior to the priming dose.

The rats had one 15 minute exploratory session and one 15 minute baseline preference test. Next, animals were conditioned using either 3 mg/kg cocaine or 5 mg/kg morphine, receiving drug and saline on the same day (at least 4 hours apart) for 3 consecutive days. The

selection of the conditioning doses reflected a balance between the desire to observe a robust conditioning response, yet achieve extinction within a reasonable amount of time, and then easily retrieve the CPP during reinstatement.

Immediately after injection, rats were confined to a particular compartment (drug-paired or saline-paired) in the CPP box for 20 minutes or 40 minutes, for cocaine and morphine respectively. A shorter conditioning time was used for cocaine because of the expectation that the rewarding properties of cocaine are relatively short-lived, and it was important that the rats were out of the boxes before the rewarding effect dissipated, and before any aversive effects emerged.

Following conditioning, preference scores were obtained exactly as for baseline preference. Individual rats which showed less than a 30-second increase in time spent in the drug-paired side during the post-conditioning test compared to their baseline preference were not included in the extinction and reinstatement data (after Mueller et al. 2000 and Ribeiro do Couto *et al.* 2005). To assess conditioning *before* exclusions, a 1-tailed Wilcoxon matched pairs signed-rank test was used (each group's preference score after drug treatment compared to its baseline).

Four cohorts of rats were used: cocaine-conditioned control (n = 20), morphine-conditioned control (n = 24), cocaine-conditioned BUP/NTX treatment (n = 16), and morphine-conditioned BUP/NTX treatment (n = 16).

For all cocaine-conditioned rats, extinction was achieved by reconditioning. This involved injection of saline followed by confinement to each compartment for 20 minutes a day for 4 days (4 hours apart). During extinction training, if a rat was placed in the drug-paired compartment in the morning session one day, it would be placed in the saline-paired compartment in the morning session the following day. Extinction was confirmed by a 15 minute free-to-explore test.

For the morphine-conditioned rats, two styles of extinction were used: a reconditioning style and a retesting style. The reconditioning style involved a saline injection with 15 minutes confinement in each compartment (as described for cocaine). The retesting style of extinction involved daily retesting, with 15 minutes per test. Both styles of extinction training are commonly used (Aguilar *et al*. 2009). Of the animals subsequently tested for reinstatement, 5 rats in the control group underwent extinction training using the

reconditioning style, and 9 underwent extinction using the retesting style. All rats in the BUP/NTX treatment group underwent extinction training using the reconditioning style.

For both extinction styles, extinction training was deemed complete if group mean preference score was <5. For the retesting style of extinction, this was taken from the average of 2 consecutive days.

Following extinction, rats were administered a priming dose of 3 mg/kg cocaine or 1.25 mg/kg morphine. The selection of the priming doses reflected a balance between the desire to observe a robust reinstatement in the control groups, and having the sensitivity to measure any possible effect of the BUP/NTX treatment on reinstatement. Furthermore, as reinstatement is a reflection of drug-seeking behaviour, the priming dose should not be so high that the animal is satiated.

The control groups received a saline injection 10 minutes prior to drug priming and the treatment groups received a BUP/NTX injection (0.3 and 1 mg/kg respectively) 10 minutes prior to drug priming. Following administration of the priming dose, rats were placed immediately into the CPP boxes and were free-to-explore during a 30-minute test.

Conditioning and reinstatement were assessed using the Friedman test followed by Dunn's multiple comparison test (each group's preference score compared to its baseline). A Mann-Whitney U test was used to compare the preference during the reinstatement test for rats that had undergone a retesting style of extinction and for rats that had undergone a reconditioning style of extinction.

Blood and brain tissue concentrations

BUP and NTX (0.3 and 1 mg/kg respectively) were administered by intraperitoneal injection. After 30 minutes, animals were killed by cervical dislocation and trunk blood and whole brains were collected and placed on ice.

Plasma sample preparation - Blood samples (~400 µl) were centrifuged at 3300 rpm for 10 minutes (Boeco, Germany), and the plasma recovered. The plasma samples were basified using an ammonium hydroxide solution of pH 10 and loaded onto a solid phase extraction (SPE) cartridge (Waters Oasis HLB) previously conditioned with 1 ml methanol and 1 ml water. The cartridge was washed with 1 ml 2 % methanol in ammonium hydroxide solution (pH 10), rinsed with water, then the drug was eluted with 440 µl 60:40 methanol: 2% acetic acid.

Brain tissue sample preparation - The brain tissue was prepared using the same SPE process as the plasma samples. Prior to the SPE process 1.8 ml water per gram of brain tissue was added to facilitate homogenisation (Tissue Master 240, OMNI International, US), the sample was centrifuged, and the supernatant fluid was collected.

BUP and norbuprenorphine LC-MS method - Separation was performed using a GeminiNX column (3µm C18 110A 50 x 2mm) from Phenomenex, maintained at 25°C, on a Shimadzu LC-2010AHT HPLC. The mobile phase was 18:82 acetonitrile: 0.1% acetic acid at a flow rate of 0.2 ml/min. 30µl of sample was injected. The sample was passed through a nylon 0.45 µm syringe filter before injection into the LC-MS. Samples were injected undiluted to analyse BUP, then diluted 1:1 with water to analyse NTX. The retention times for BUP and norbuprenorphine were 9 and 3 minutes, and the masses per charge were 468 and 414. Standards were prepared in SPE eluent. Limit of quantitation was 0.18 ng/ml for BUP and 0.8 ng/ml for norbuprenorphine.

NTX and 6ß-naltrexol LC-MS method - The method for analysis of NTX and 6ß-naltrexol was adapted from Valiveti *et al.* (2004). Separation was performed using a Symmetry column (5µm C18 110A 150 x 2.1 mm) from Waters, maintained at 23°C, on a Shimadzu LC-2010AHT HPLC. The mobile phase was 12:88 acetonitrile: 0.1 % ammonium acetate at a flow rate of 0.25 ml/min. 30µl of sample was injected. The retention times for NTX and 6ß-naltrexol were 4 and 3 minutes, and the masses per charge were 342 and 344. Standards were prepared in SPE eluent. Limit of quantitation was 3.8 ng/ml for NTX and 0.28 ng/ml for 6ß-naltrexol.

Receptor occupancy calculations

From the observed plasma and brain concentrations, receptor occupancy levels of each drug could be estimated using affinity values (equilibrium dissociation constants). Calculations of relative receptor occupancy were derived using the equation below (Rang & Dale 2016).

Equation 3.3

$$\% \text{ occupancy of drug A} = 100 * ([A]/KA) / (([A]/KA) + ([B]/KB) + 1)$$

where [A] = the tissue concentration of drug A, [B] = the tissue concentration of drug B, K_A = the equilibrium dissociation constant of drug A, and K_B = the equilibrium dissociation constant of drug B.

The equilibrium dissociation constants for BUP and NTX at the mu receptor were 0.41 nM and 1.26 nM respectively (Ridzwan 2012). The equilibrium dissociation constants at the kappa receptor for BUP and NTX were 0.8 nM and 0.4 nM respectively (Toll *et al*. 1998).

3. Results and Discussion

The aim of this chapter was to assess the ability of the BUP/NTX combination to reduce the likelihood of relapse following a cocaine or morphine prime. Firstly, the tail withdrawal method, an assay of analgesia, was used to confirm that 0.3 mg/kg BUP with 1.0 mg/kg NTX could block the mu agonism produced by 10 mg/kg morphine. Subsequently, the CPP-extinction-reinstatement method was used to measure the effect of the BUP/NTX combination on drug-primed reinstatement to morphine and to cocaine. The results are presented below.

Measuring mu receptor antagonism of a BUP/NTX combination

Prior to commencing behavioural work, the ability of 0.3 mg/kg BUP and 1.0 mg/kg NTX to block the mu receptor agonism of 10 mg/kg morphine was measured (figure 3.2).

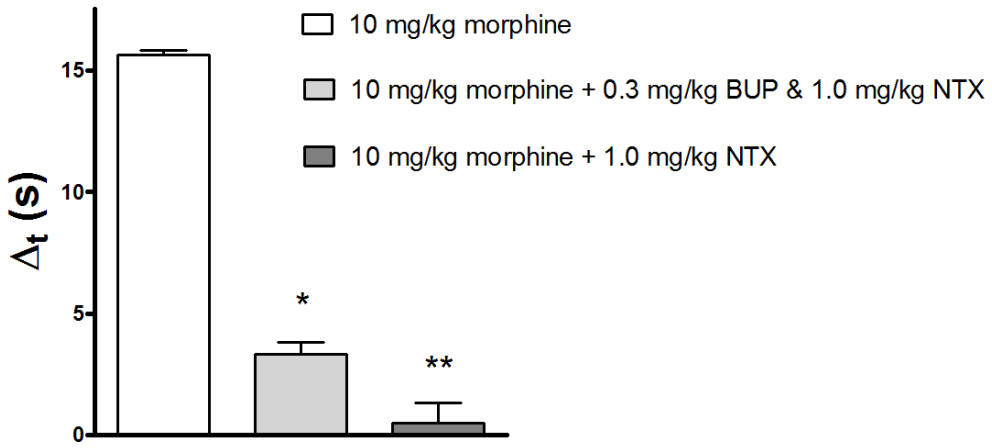

Figure 3.2: Tail withdrawal assay to measure antagonism of morphine by NTX, with and without BUP. Data shows measurements taken 30 minutes after administration of 10 mg/kg morphine, and 60 minutes after administration of BUP and/or NTX (n = 5, 4, 7). Baseline values were in the range 4 - 5 seconds. Mean + SEM; * indicates significantly different ($p < 0.05$) vs. morphine alone group; ** indicates significantly different (one-way ANOVA with the Bonferroni post-test, $p < 0.05$) vs. morphine control group and vs. 0.3 mg/kg BUP and 1.0 mg/kg NTX group.

As expected, 10 mg/kg morphine elicited a measurable analgesia. Clear mu receptor antagonism was observed following administration of BUP/NTX (morphine-induced analgesia decreased from 16 ± 0 to 3 ± 1 seconds, mean ± SEM). Administration of NTX alone blocked morphine-induced analgesia to a greater extent than the BUP/NTX

combination. This result provided justification to test the combination at these doses in the CPP behavioural model.

CPP extinction and reinstatement model

The CPP-extinction-reinstatement method was used to observe drug-primed reinstatement in cocaine- and morphine-conditioned rats, and the effects thereon of treatment with a BUP/NTX combination. Table 3.1 shows the number of rats used in the experiments, the number of rats excluded, and the time taken to reach extinction. Conditioning was statistically significant in each cohort before individual rats were excluded for insufficient conditioning.

Table 3.1: Number of rats used in the experiments, and excluded at each stage, and time taken to reach extinction.

	Cocaine control cohort	Cocaine-cohort with BUP/NTX	Morphine control cohort	Morphine-cohort with BUP/NTX
At start	20	16	24	16
Excluded for baseline preference	0	2	2	1
Excluded for insufficient conditioning	8	5	8	7
Time taken to reach extinction	1 week	1 week	1 week or 12 days	1 week

It was observed that in the control cocaine-conditioned group, preference for the drug-paired compartment emerged at 13-15 minutes of the 30 minute reinstatement test period (see figure 3.3, left panel). Mueller and Stewart (2000) also observed an emergence of drug seeking during the reinstatement test (see figure 3.4, left panel) following a cocaine drug prime in cocaine-conditioned rats, though over a shorter period of time.

Unlike the cocaine-conditioned rats, the morphine-conditioned rats showed the greatest preference during the first 15 minutes of the reinstatement test (figure 3.3, right panel). This is in contrast to Mueller *et al.* (2002) who saw an emergence of drug-seeking in morphine-conditioned rats at around 20 minutes (figure 3.4 right panel). The data presented here are more comparable with Bozarth (1987) who showed that rats reinstated to seek heroin decreased their drug-seeking behaviour over the duration of a 15 minute test. The data from 0-30 minutes of the reinstatement test were used for the cocaine-conditioned rats, and the data from 0-15 minutes of the test were used for the morphine-conditioned rats.

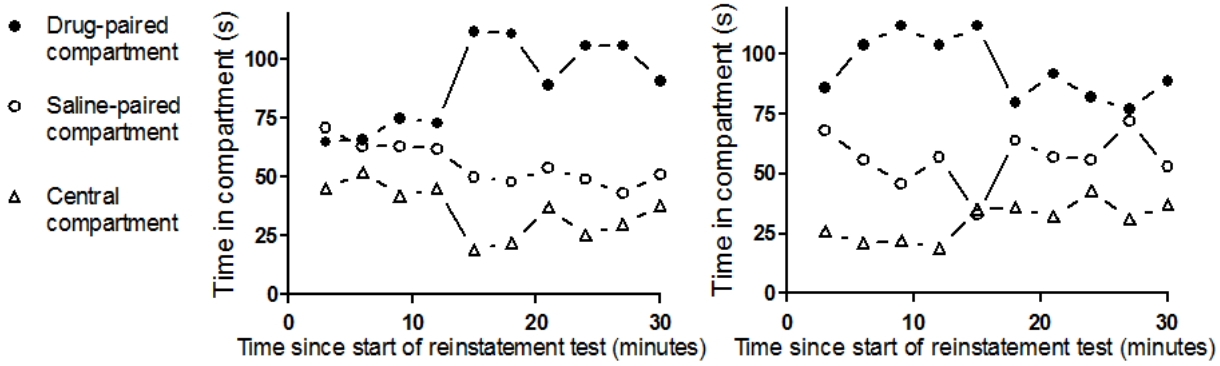

Figure 3.3: Preference for the 3 CPP compartment types across the 30 minutes of the reinstatement test. **Left**: Following a 3.0 mg/kg cocaine prime. **Right**: Following a 1.25 mg/kg morphine prime. Data shown in 3 minute bins. Error bars not shown for clarity.

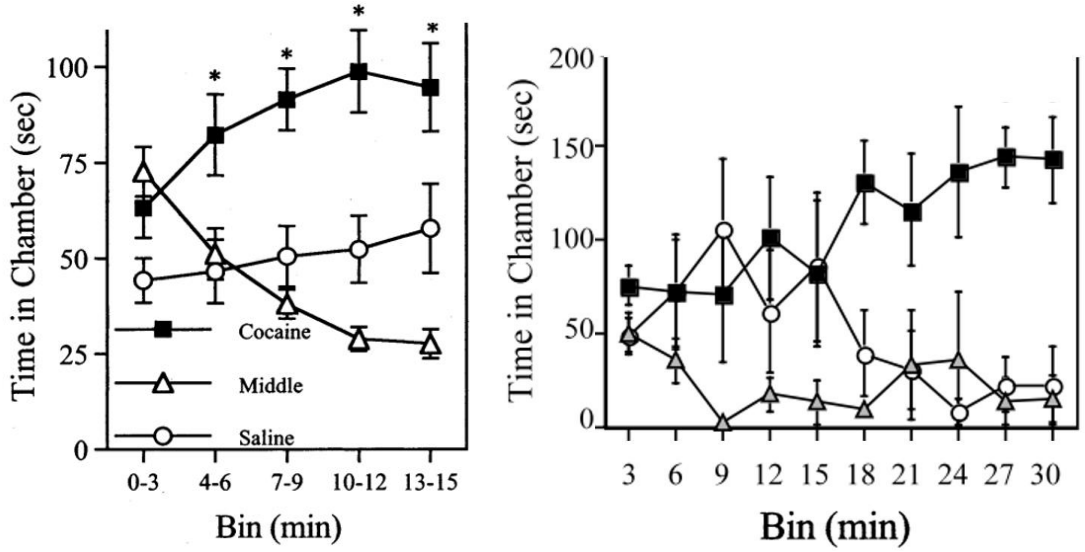

Figure 3.4: Preference for the 3 CPP compartment types across the reinstatement test. **Left**: following a 5.0 mg/kg cocaine prime (taken from Mueller and Stewart 2000). **Right**: following a 2.5 mg/kg morphine prime (taken from Mueller *et al*. 2002). Data shown in 3 minute bins. Mean ± SEM. Figures reproduced with kind permission from Elsevier.

Figure 3.5 (top panel), figure 3.6 (top panel), and table 3.2 show that for the control groups, conditioning, extinction and then reinstatement were successfully achieved. The preference post-conditioning and following a drug prime was significantly different from baseline for both drugs. As there was no significant difference in preference score during the reinstatement test between the morphine-conditioned rats, which underwent the two styles of extinction training used (reconditioning 11 ± 7, n = 5; retesting 19 ± 9, n = 9; mean ± SEM), pooled data are shown.

Achieving conditioning, extinction and drug-primed reinstatement using the conditioned preference method has been previously reported for morphine (Parker & McDonald 2000) and cocaine (Mueller & Stewart 2000). The novel aspect of the work reported here is the effect of the BUP/NTX combination on reinstatement; these results are shown in figure 3.5 (bottom panel), figure 3.6 (bottom panel), and table 3.2.

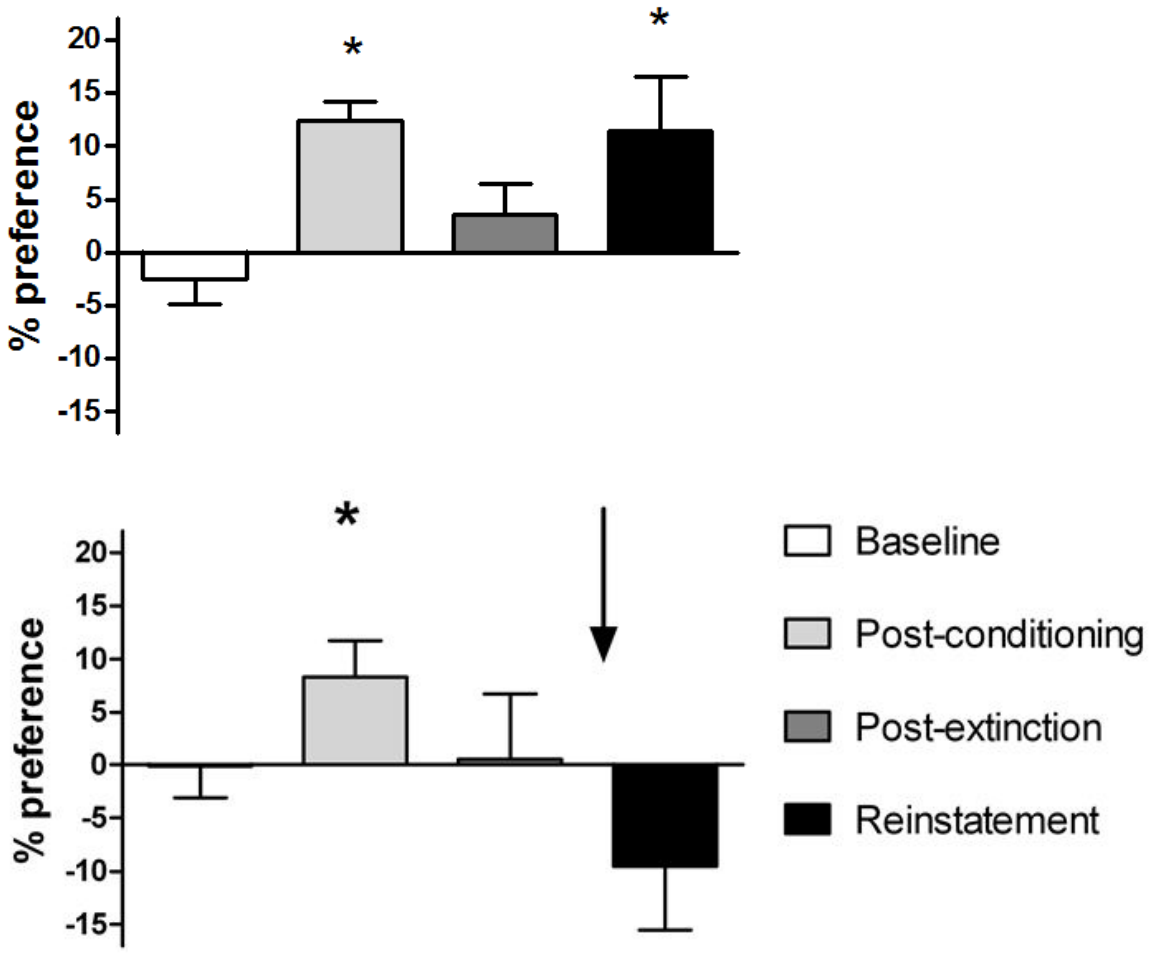

Figure 3.5: Effect of BUP/NTX treatment on reinstatement to cocaine-seeking. Preference scores (left to right in each panel: baseline, post-conditioning, post-extinction and drug-primed reinstatement). Drug prime was 3 mg/kg cocaine. **Top panel** cocaine-conditioned control group (n = 12); **Bottom panel** cocaine-conditioned BUP/NTX treated group (n = 9). Mean + SEM; * indicates significantly different from baseline (Friedman test followed by Dunn's multiple comparison test, each group's preference score compared to its baseline, $p < 0.05$). Arrows indicate that BUP/NTX treatment (0.3 mg/kg/1.0 mg/kg) was administered 10 minutes prior to the priming dose.

For the BUP/NTX treated groups, conditioning and extinction were successfully achieved. The effect of the combination on drug-primed reinstatement in both the cocaine- and morphine-conditioned rats is clear. Preference during reinstatement test was not significantly different from baseline for either drug. In the cocaine-conditioned rats, the BUP/NTX treatment completely blocked reinstatement (preference score of -9 ± 5 mean ±

SEM compared to 11 ± 5 in the control group). In the morphine-conditioned rats, the BUP/NTX treatment attenuated the preference observed following administration of a drug prime (preference score of 6 ± 10, mean ± SEM compared to 16 ± 6 in the control group).

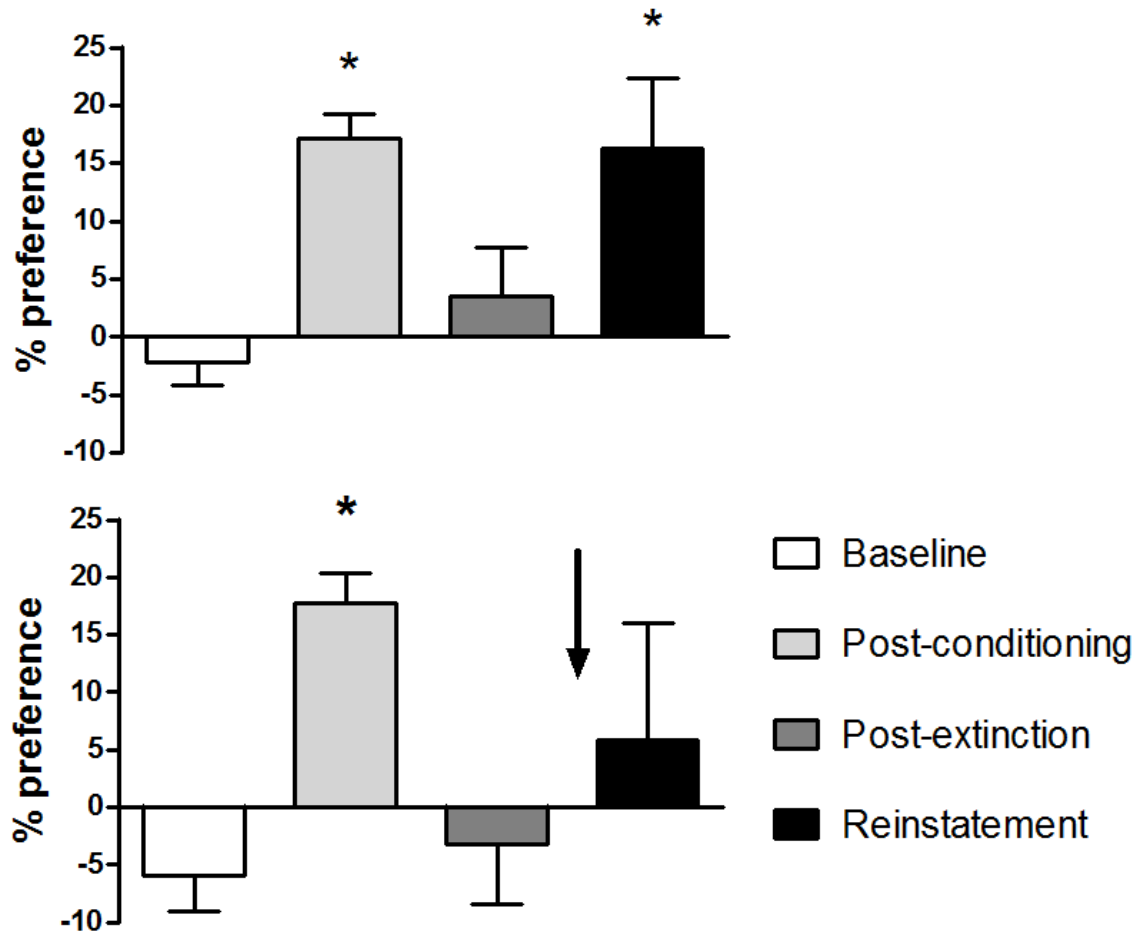

Figure 3.6: Effect of BUP/NTX treatment on reinstatement to morphine-seeking. % preference of rats (left to right in each panel: baseline, post-conditioning, post-extinction and drug-primed reinstatement). Drug prime was 1.25 mg/kg morphine. **Top panel** morphine-conditioned control group (Mean + SEM of n = 14). **Bottom panel** morphine-conditioned BUP/NTX treated group (Mean + SEM of n = 8); * indicates significantly different from baseline (Friedman test followed by Dunn's multiple comparison test, each group's preference score compared to its baseline $p < 0.05$). Arrows indicate that BUP/NTX treatment (0.3 mg/kg/1.0 mg/kg) was administered 10 minutes prior to the priming dose.

Table 3.2: Time spent in the drug-paired compartment at the different stages of the CPP experiment (n = 12, 9, 14, 8). As the test session is 15 minutes, a score of 450 indicates equal time spent in each compartment. Data shown as mean ± SEM.

Time in drug-paired compartment (s)	Cocaine control cohort	Cocaine-cohort with BUP/NTX	Morphine control cohort	Morphine-cohort with BUP/NTX
Baseline	427 ± 20	449 ± 27	431 ± 18	396 ± 28
After conditioning	562 ± 16	547 ± 39	604 ± 19	610 ± 23
After extinction	482 ± 26	455 ± 55	482 ± 38	421 ± 47
After reinstatement	553 ± 47	365 ± 54	596 ± 55	502 ± 92

The work presented here is a clear advance with respect to previous knowledge. There is preclinical data showing that BUP on its own (Kosten *et al*. 1991; Suzuki *et al*. 1992; Comer *et al*. 1993) and NTX on its own (Bilsky *et al.* 1992; Suzuki *et al.* 1992) can reduce the ability to *acquire* a conditioned place preference to cocaine. However, here, the ability of BUP and NTX to block drug-primed reinstatement to cocaine-seeking following extinction was demonstrated. Furthermore, the two compounds were administered as a combination that by itself induces neither conditioned place preference nor aversion. Importantly, the observed reduction in cocaine-seeking implies that the decrease in cocaine use reported in clinical trials (Rothman *et al*. 2000; Gerra *et al.* 2006) was probably not a simple consequence of reduced heroin use.

The blocking of drug-primed reinstatement to morphine by a BUP/NTX combination has not been shown before, but is expected and can be explained by pharmacological blockade of the mu agonist effect of morphine. In contrast, the novel observation that a BUP/NTX combination can block drug-primed reinstatement to cocaine has no simple explanation. Some hypotheses are given below.

1. It is possible that the overall mu antagonist activity of the combination could prevent reinstatement by blocking endogenous opioids that are indirectly activated by cocaine (Soderman & Unterwald 2008; Le Merrer *et al.* 2009), or that the mu activity of the combination somehow normalises the dysregulated opioid system observed following cocaine use (Yoo *et al.* 2012).

2. It generally expected that kappa antagonism should not influence drug-primed reinstatement to cocaine (Beardsley *et al.* 2005), but should only reduce stress-induced reinstatement to cocaine (Beardsley *et al.* 2005; Carey *et al.* 2007; Land *et al.* 2009). However, Wee *et al.* (2012) argues that the negative mood state that occurs following chronic use of cocaine is caused by kappa overdrive, and that kappa antagonsim could reduce cocaine use in general, regardless of whether in response to stress or to a drug prime.

3. The third receptor that the combination (only BUP) binds to is NOP. There is convincing evidence that NOP is anti-addictive (Marquez *et al.* 2008a; Rutten *et al.* 2011a), for substances of abuse including cocaine (Kotlinska *et al*. 2002; Marquez *et al.* 2008b; Bebawy *et al.* 2010). However, the affinity of BUP for the NOP receptor measured *in vitro* is relatively low; equilibrium dissociation constants from 8.4 nM (Wnendt *et al.* 1999) to 77.4 nM (Spagnolo *et al.* 2008) have been reported. Therefore it is not yet known what dose of BUP would be required for NOP agonism to become relevant to drug-seeking behaviour.

4. The last explanation proposed for the observed blocking of drug-primed reinstatement to cocaine by the BUP/NTX combination is that it is an experimental artefact. It is conceivable that BUP/NTX could somehow prevent the recall of the association between the cocaine reward and the cocaine-paired compartment. In this case, reduced time spent in the drug-paired compartment during the reinstatement test would not indicate a true reduction in drug-seeking. Overall, this explanation for the observed result is considered unlikely; neither BUP nor NTX are known to effect spatial memory retrieval.

Finally, a caveat of the findings of this chapter is that the BUP/NTX combination was given acutely, within minutes of the priming dose. Obviously as a relapse prevention therapy it would be administered chronically.

Receptor occupancy of BUP and NTX from blood and brain concentrations

When the BUP/NTX combination was administered 10 minutes before a cocaine or morphine drug prime, reinstatement was blocked. In order to establish the plasma and brain concentrations of BUP and NTX that would have been present during the reinstatement test, a separate cohort of rats were administered with 0.3 mg/kg BUP and 1.0 mg/kg NTX, and drug concentrations after 30 minutes were measured. The primary metabolites of BUP and NTX (norbuprenorphine and 6β-naltrexol) were also assayed but were below the limit of quantitation in plasma and brain samples. From the BUP and NTX concentrations the mu and kappa receptor occupancies of each drug could be estimated using their equilibrium

dissociation constants (table 3.3). The amount of drug bound to the receptor has an insignificant effect on circulating concentrations; receptor occupancy calculations for mu and kappa were therefore considered separately.

Table 3.3: Plasma and brain concentrations of BUP and NTX and estimated receptor occupancies. BUP and NTX were administered simultaneously intraperitoneally. Plasma and brain samples were taken 30 minutes later and BUP and NTX levels measured. Using the plasma and brain concentrations, and literature affinity values of both BUP and NTX, mu and kappa receptor occupancy levels were determined. All data shown as mean ± SEM.

	BUP			NTX		
	Observed concentration (nM)	Predicted occupancy of mu receptor (%)	Predicted occupancy of kappa receptor (%)	Observed concentration (nM)	Predicted occupancy of mu receptor (%)	Predicted occupancy of kappa receptor (%)
Plasma	56 ± 4	38 ± 3	8 ± 1	285 ± 19	62 ± 4	91 ± 6
Brain	83 ± 14	45 ± 8	12 ± 2	313 ± 19	55 ± 3	88 ± 5

These data suggest that at the time of the drug prime, more than 95% of available mu receptors were occupied either by BUP or NTX. This is consistent with Greenwald *et al.* (2014) who found that when more than 90 % of mu receptors were occupied (following administration of BUP in man) the subjective effects of “higher-than-usual” doses of abused opioids were blocked.

The optimal relative receptor occupancies for this therapeutic application are not yet known. The results shown here indicate that when ~45% of the mu receptors in the brain are occupied by BUP and ~55% are occupied by NTX, neither aversion nor reward is experienced (chapter 2). Furthermore, at the doses administered here, these relative receptor occupancies were sufficient to block a cocaine or morphine prime.

Lastly, these data indicate that more than 95% of available kappa receptors in the brain were occupied by BUP or NTX. Therefore, as well as blocking mu receptors, the combination of BUP and NTX used here also acts as a functional kappa receptor antagonist.

A caveat of the receptor occupancy calculations is that only absolute brain levels were measured rather than free concentrations of each drug. A high proportion of the compounds may be in the lipid compartment and unavailable for receptor binding. The brain levels measured here were far in excess of the equilibrium constant values, so using free concentrations would be unlikely to have an impact on total receptor occupancy. However, if the proportion of drug bound was very different for BUP and NTX, this would of course be expected to influence the predictions of relative receptor occupancies.

As mentioned briefly in chapter 2, it is not known to what extent the results observed here in a rat model will translate into a clinical situation. Some ways in which the CPP-extinction-reinstatement model lacks construct validity are presented below.

Firstly, the timeframe of the CPP method used here is vastly different from the clinical situation. In this work, the rats received 3 doses of cocaine, or 3 doses of morphine; this is clearly not equivalent to frequent use of a drug of abuse over many years. Similarly, the drug-free extinction period here was 1 or 2 weeks, in contrast to the years of abstinence that can precede relapse in man.

A second important difference between the reinstatement model in rodents and the clinical situation is that extinction occurs for different reasons (abstinence is imposed or abstinence is chosen). Epstein *et al.* (2006) believes this to be the single biggest conceptual difference between the animal and human situation, though it is not yet known if this difference translates into reinstatement/relapse outcomes. Likewise, a human self-initiates drug use in the event of a lapse, whereas the rodent is administered the priming dose by the experimenter (Aguilar *et al.* 2009).

Lastly, the magnitude of relapse/reinstatement response is believed to decrease over time in humans, but not in rodents (Epstein *et al.* 2006). As for the results of chapter 2, the findings presented here would ideally be confirmed in human patients.

4. Conclusions of the chapter

In summary, it has been shown that a combination of 0.3 mg/kg BUP and 1.0 mg/kg NTX, administered intraperitoneally in Sprague Dawley rats, results in high occupancy levels of both mu and kappa receptors, and so acts as a functional mu/kappa receptor antagonist. It was demonstrated that 0.3 mg/kg BUP and 1.0 mg/kg NTX blocked drug-primed reinstatement to cocaine-seeking, and attenuated drug-primed reinstatement to morphine-

seeking. These data add to the growing evidence that a BUP/NTX combination may be effective in a polydrug abuse situation. In chapter 4, the delivery of BUP and NTX via the transdermal route will be addressed.

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Chapter 4: Feasibility of iontophoretic transdermal delivery of buprenorphine and naltrexone

1. Introduction

NTX is currently available as an oral tablet (as Revia®), while BUP is formulated as a sublingual tablet (as Subutex®). Delivering NTX and BUP in a combination oral tablet would be difficult because of BUP's low oral bioavailability due to first pass effect (McQuay & Moore 1995). Unfortunately, delivery of the combination as a sublingual tablet might be difficult because of NTX's low bioavailability by this route, probably due to the poor permeability of the drug (Lewis & Lloyd-Jones 1987).

Transdermal formulation could provide an advantageous platform for the delivery of the two drugs together. Transdermal delivery could result in reduced opportunity for abuse/diversion, increased patient adherence, and avoidance of daily fluctuations in plasma concentrations. Iontophoresis offers greater control over delivery rates compared to passive delivery (Harper Bellantone *et al.* 1986). Furthermore, it is expected to be a more suitable delivery approach for NTX, which has low passive fluxes (Hammell *et al.* 2004 & 2005), possibly due to its relative hydrophilicity. The objectives of this chapter were to determine if BUP and NTX could be delivered together by transdermal iontophoresis, and to assess their relative delivery efficiencies.

Mechanisms of iontophoretic transport

During iontophoresis, molecules move across the skin via three mechanisms: electrorepulsion (ER), electroosmosis (EO) and passive diffusion.

1. *Electrorepulsion*

The movement of ions during iontophoresis is driven by the electrochemical reactions at the electrodes. The reactions shown here (Equation 4.1) take place when silver/silver chloride electrodes are used; these are considered the best option for use in iontophoresis (Phipps *et al*. 1989).

Equation 4.1

$$\text{anode:} \quad Ag + Cl^- \rightarrow AgCl_{(s)} + e^-$$
$$\text{cathode:} \quad AgCl_{(s)} + e^- \rightarrow Ag + Cl^-$$

When current is passed, electroneutrality is maintained by movement of ions across the skin (Phipps *et al.* 1989). An anion can move toward the anode, or a cation can move toward the cathode. Figure 4.1 illustrates the movement of ions in an iontophoretic system.

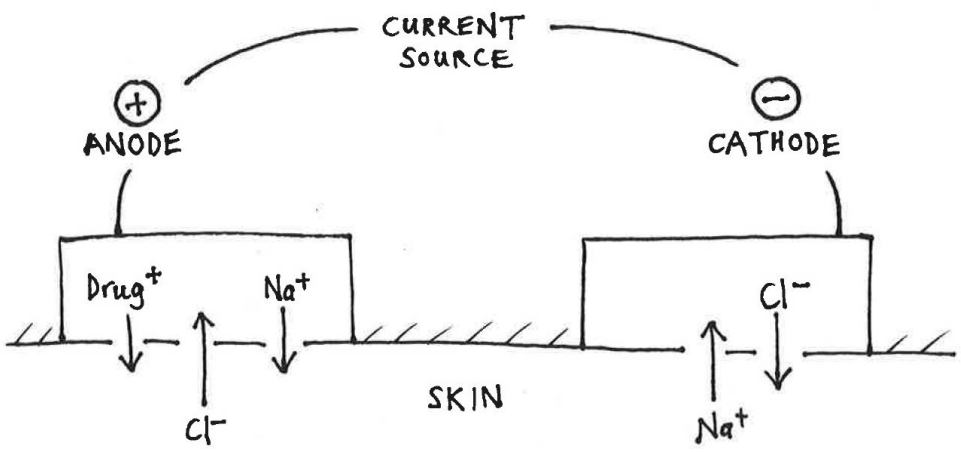

Figure 4.1: Schematic representation of the movement of ions in an iontophoretic patch system on the skin. Redrawn from Kalia *et al.* 2004. In this example, the drug is positively charged, therefore is delivered from the anode. Ions present at the anode and cathode compartments would be from a buffer for example.

For every one electron that moves round the circuit, one (singly-charged) ion will move, thus there is finite movement of ions. Therefore, competition to carry the current exists between co-ions (ions of same polarity) and between counter-ions (ions of opposite polarity) (Burnette & Ongpipattanakul 1987; Pikal & Shah 1990a & b). Which ions in the system move is determined by their relative mobilities, and by their relative abundances (Riviere & Heit 1997; Mudry *et al.* 2007). Smaller ions (smaller hydrodynamic radius) are more mobile than larger ions (Riviere & Heit 1997). Singly-charged ions are more efficiently delivered than multiply-charged ions (Phipps *et al*. 1989); for one doubly-charged ion to be transported, two electrons must pass round the circuit. Furthermore, under normal circumstances, the skin is negatively charged, so all things being equal, cations will move across the skin in larger numbers than anions (Burnette & Ongpipattanakul 1987). Note the contrast between the situation of iontophoresis and the situation of passive delivery. In passive delivery, the flux of drug A would not be expected to be influenced by the concentration of drug B in the donor solution. In iontophoresis, two drugs of the same sign of charge (both cations or both anions) are expected to compete for charge carrying. This is a fundamental difference between passive delivery and iontophoresis (Riviere & Heit 1997).

How good an ion is at competing for charge (and therefore how efficiently it can be delivered) can be quantified by calculating its transport number. Transport number is simply the fraction of the total current transported by that ion, and therefore has a theoretical

maximum of 1. For example, if for every 4 electrons that pass round the circuit, one (singly-charged) drug ion crosses the skin, the transport number of the drug is 0.25. In reality, transport numbers for drug molecules will generally be small, with the vast majority of the charge being carried by other smaller, more mobile ions present in the system.

Competition between co-ions in the system means that increasing the molar fraction of an ion results in increasing flux of that ion (Marro *et al*. 2001c; Kalia *et al.* 2004, Phipps & Gyory 1992). This is illustrated in figure 4.2 where, as the concentration of the co-ion (in this case Na+, from sodium chloride) in the donor decreases, the flux (and therefore the transport number) of lidocaine increases.

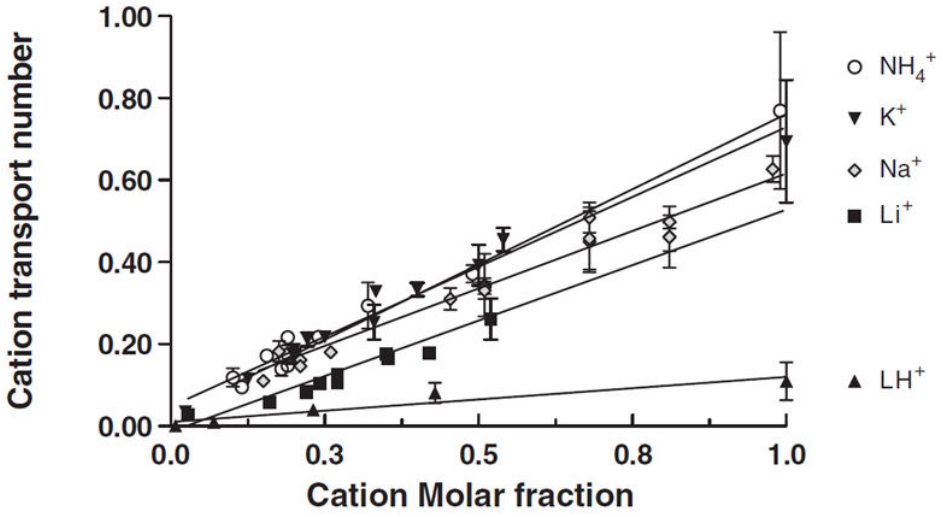

Figure 4.2: Transport number as a function of molar fraction (Mudry *et al.* 2006a, data generated *in vitro* using pig skin). LH+ is lidocaine hydrochloride. Lidocaine is delivered at lower efficiency than the small inorganic ions because it is larger size (234 Da) and therefore lower mobility. Reproduced with kind permission from Elsevier.

Clearly, the highest transport number will be observed in the 'single-ion situation', when the drug is the only ion of its polarity (cation or anion) in the donor compartment and the molar fraction is therefore 1 (Mudry *et al.* 2006a). Note that in the single-ion situation, changing the concentration of the drug in the donor will *not* affect flux (because molar fraction is always 1). This is important because it means that, in contrast to passive delivery, maximal flux can be achieved with drug concentrations below saturation (Marro *et al.* 2001b). That said, the single-ion situation is unlikely to occur in a commercial product, because stabilisers, preservatives, buffers, or viscosity agents are likely to be present.

The pH of the donor affects ER by determining the ionisation of the drug as obviously, only ionised drug is transported by ER (Merino *et al.* 1999; Lopez *et al.* 2001). Therefore, judicious selection of pH can, to a certain extent, be used to increase the proportion of

charge that is carried by the drug molecules, and thus the efficiency of drug delivery. Other strategies to increase the flux of drug include choosing larger (therefore less mobile) buffer co-ions, and increasing the molar fraction of the drug in the donor solution. Increasing the molar fraction of the drug can be achieved by increasing the drug concentration, or by decreasing background co-ion concentrations. Competition between drug ions and counter-ions is not as easy to manipulate. In the case of a drug delivered from the anode, the most important counter-ion is subdermal chloride (Phipps *et al.* 1989); in the case of a drug delivered from the cathode, the most important counter-ion is subdermal sodium. Clearly there is no control over the subdermal compartment, and this places a limit on how efficient iontophoresis can ever be.

The last point to make about ER, and the most intuitive, is that iontophoretic flux increases proportionally with current intensity (Kalia *et al.* 2004), as predicted by Faraday's law.

Equation 4.2

$$J = \frac{I \times t}{z \times F}$$

J is flux, I is current intensity, t is transport number, z is the valence of the ion, and F is Faraday's constant.

2. Electroosmosis

When current is passed across the skin, a convective solvent flow, known as EO, is established (Pikal & Shah 1990a & b; Burnette & Ongpipattanakul 1987; Luzardo-Alvarez *et al.* 1998). This solvent flow occurs due to the skin; the net negative charge of the skin under normal conditions makes it permselective to cations. EO occurs in the anode-to-cathode direction, and so drug delivery from the anode is 'helped' by EO. EO acts on all molecules present at the anode, including uncharged ones. The volume of EO solvent flow is dependent on buffer type, ionic concentration, and pH (Tamada & Comyns 2005), but the volume of convective solvent flow that occurs during iontophoresis is generally in the region of 1 to 2 µl/h/cm^2 (Kim *et al*. 1993; Marro *et al.* 2001a; Dubey & Kalia 2014). It is possible to directly measure movement of water between compartments of an iontophoretic cell either by using a cell linked to a capillary tube (Pikal & Shah 1990a) or by measurement of tritiated water (Kim *et al.* 1993). However, it is more common to use the flux of a marker such as mannitol or acetaminophen as an indicator of 'apparent solvent flow'. These molecules are suitable as markers because they are uncharged and have negligible passive flux (Dubey & Kalia 2014).

The relative contributions of ER and EO to total iontophoretic flux depend on the permeant, and upon the experimental conditions (Merino *et al.* 1999; Tamada & Comyns 2005). Obviously, EO is likely to be the largest contributing factor to flux for large uncharged molecules, whilst ER is likely to be the largest contributing factor to flux for small, charged ions (Riviere & Heit 1997; Marro *et al.* 2001b). Unlike ER, the effect of molecular size on electroosmotic transport is not fully established, yet there is some experimental evidence suggesting that electroosmotic transport decreases as molecular weight increases (Yoshida & Roberts 1993, Djabri *et al*. 2015).

Although under normal conditions EO occurs in the anode-to-cathode direction (Delgado-Charro & Guy 1994; Burnette & Ongpipattanakul 1987; Pikal & Shah 1990a), the direction of the solvent flow can change if the charge on the skin is altered. The charge on the skin can be altered either by changing the pH (Kim *et al.* 1993; Merino *et al.* 1999; Marro *et al.* 2001a), or by accumulation of cations in the skin (Delgado-Charro & Guy 1995; Schuetz *et al.* 2005; Dubey & Kalia 2014). The effect of pH will be discussed first.

As mentioned, under physiological conditions the skin carries a net negative charge, believed to correspond to acid residues. As the pH is lowered, these groups become less ionised and the overall negative charge is less. The isoelectric point of human skin (the pH at which the skin carries no net charge), is close to pH 4.5 (Marro *et al.* 2001a). At pHs below the isoelectric point, the skin carries a net *positive* charge, and the direction of EO is reversed. This is illustrated in figure 4.3, which shows the effect of pH on anode-to-cathode EO and on cathode-to-anode EO.

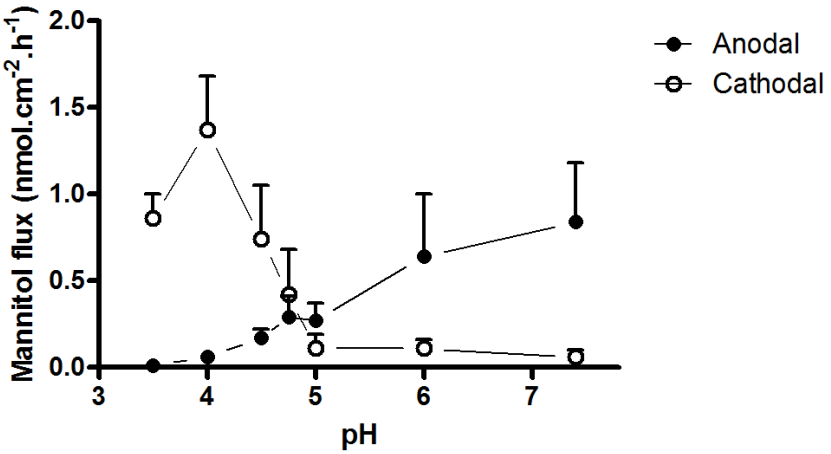

Figure 4.3: Effect of pH on EO. The magnitude of mannitol flux (mean ± standard deviation) after 6 h of iontophoresis across human skin *in vitro* depends on the pH. The experiments were carried out in symmetrical conditions (the same background electrolyte was used on both sides of the skin). Transport in the anode-to-cathode direction (filled circles) is compared to that in the cathode-to-anode direction (open circles). Redrawn from Marro *et al.* (2001a).

Interestingly, it can be seen that EO occurs simultaneously in the anode-to-cathode direction and in the cathode-to-anode direction. To explain this observation, it has been suggested that there are (at least) 2 populations of channels, differing in polarity of charge, or pore size, or charge density (Pikal 2001). However, there is no direct experimental evidence for this hypothesis.

The second mechanism by which the charge on the skin and thus the direction of EO can be altered is by accumulation of cations in the skin itself. Figure 4.4 shows a clear and dramatic reversal of the direction of EO, due to an accumulation of nafarelin in the skin.

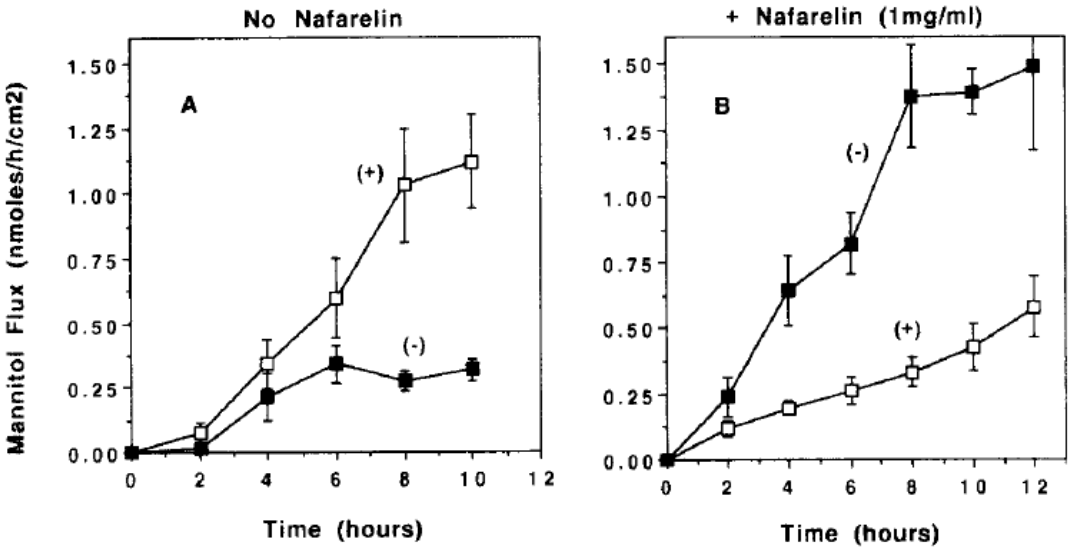

Figure 4.4: Anodal (□) and cathodal (■) mannitol flux (mean ± standard deviation) in the absence or presence of the cationic peptide nafarelin in the anodal compartment was measured across hairless mouse skin. Taken from Delgado-Charro & Guy (1995) and reproduced with kind permission from Elsevier.

3. Passive diffusion

Of course, passive diffusion still occurs during iontophoresis (Riviere & Heit 1997) though obviously in a system optimised for iontophoresis the contribution to total flux from passive diffusion is likely to be small. A typical observation *in vitro* is that after a period of iontophoresis, passive flux is often higher than pre-iontophoresis levels (Pikal & Shah 1990b; Thysman *et al*. 1994b; Santi & Guy 1996b), however, the relevance of this effect *in vivo* is unclear.

Passive fluxes across skin from a saturated aqueous solution can be usefully predicted from the logP and molecular weight of the permeant (Potts & Guy 1992). However, prediction of iontophoretic flux is not as straightforward (Yoshida & Roberts 1992). Prediction of the

electrorepulsive component of iontophoretic flux of ions is possible if their charge, and their concentrations and mobilities in the skin are known (Mudry *et al.* 2006a). Obviously this latter information is not readily available. Furthermore, the magnitude of the solvent flow, and its contribution to total iontophoretic transport is not easily predicted. Consequently, total iontophoretic flux is generally established empirically. In this chapter the influence of some formulation and iontophoretic parameters on the delivery of BUP and NTX was examined, and is reported in the following four sections:

- Section A: Feasibility study
- Section B: Effect of pH on iontophoresis of NTX and BUP
- Section C: Effect of drug concentration in the donor on iontophoresis of NTX and BUP
- Section D: Effect of current density on iontophoresis of NTX and BUP

2. Methods and materials

Materials

Buprenorphine hydrochloride (BUP) was from Reckitt and Colman (UK). Naltrexone hydrochloride (NTX), acetaminophen (ACM), Tris base, Tris hydrochloride, sodium chloride, silver, silver chloride and trifluoroacetic acid were from Sigma (UK). Organic solvents were from Fisher (UK). The dorsal pig skin was collected from a local abbatoir, dermatomed (Zimmer®, US), to a nominal thickness of 750 µm, frozen within 24 hours of slaughter, and thawed before use.

Iontophoresis

Excised dorsal pig skin was placed across the aperture of a side-bi-side cell and held together in a clamp (figure 4.5). The cell had two compartments, enabling the outer surface of the skin to be bathed in donor solution and the subdermal side to be bathed in receiver solution. Both compartments were stirred by magnetic bars. A power supply (Kepco, UK) was connected to a silver/silver chloride anode and cathode prepared in-house (Green 1996). The anode was in the donor compartment and the cathode was in the receiver compartment. A fixed current was passed for 6 hours, during which the receiver was sampled every hour for analysis by high performance liquid chromatography (HPLC). In a typical experiment 3 cells were run at the same time, and connected to the power supply in series.

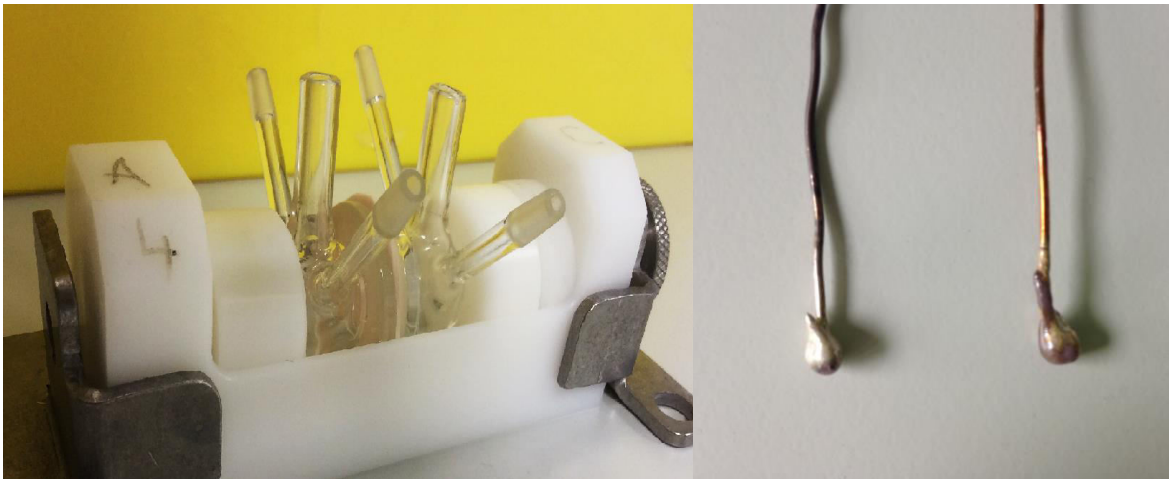

Figure 4.5. Photographs of a glass diffusion cell in a clamp, and a pair of Ag/AgCl electrodes. The surface of the electrode on the left is largely silver, so can be used as an anode; the surface of the electrode on the right is largely silver chloride so can be used as a cathode. The electrodes are placed in the vertical ports of the diffusion cell. The lateral ports of the diffusion cell can be used to sample the receiver compartment.

Section A: Feasibility study

The side-bi-side cells used in this section allowed 0.95 cm^2 of skin to be exposed and available for drug transport. The volume of the donor solution was 1.3 ml, the volume of the receiver solution was 1.8 ml. The donor solution was Tris/TrisHCl buffer pH 6 (60 mM Tris hydrochloride chloride & 0.4 mM Tris base), containing 1 mg/ml BUP with either 0, 0.55, 5.5, or 55 mg/ml NTX. The receiver solution was phosphate buffered saline pH 7.4 (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per litre of water). A direct, constant current of 0.38 mA was passed. Each hour 1 ml of receiver solution was removed for analysis and replaced with fresh buffer. A passive (no current) experiment was also performed during which the receiver was only sampled at 3 and 6 hours.

Four different donor solutions were tested. The concentration of NTX was varied, while the concentration of BUP was held constant. The table below describes the experiments presented in this section.

Table 4.1: Summary of the initial experiments carried out to assess the relative transdermal fluxes of NTX and BUP, and the effect of the concentration of NTX in the donor solution.

NTX in donor (mg/ml)	BUP in donor (mg/ml)	Donor	Receiver	Current intensity (current density)
0	1.0	60 mM Tris HCl, pH 6	Phosphate buffered saline, pH 7.4	0.38 mA (0.4 mA/cm^2)
0.55				
5.5				
55				
0.55				Passive

Section B: Effect of pH

The side-bi-side cells used in this section allowed 0.95 cm^2 of skin to be exposed and available for drug transport. The volume of both the donor and receiver solutions was 3.5 ml. The donor solution was 0 or 1 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl (pH was adjusted to 4, 5 or 6 with Tris base, NaOH or HCl). The receiver solution was 60 mM TrisHCl (pH adjusted to 4, 5 or 6 with Tris base, NaOH or HCl). For pH 6 only, a passive (no current) experiment was also performed; during which the receiver was only sampled at 3 and 6 hours.

A fixed current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3 times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. The donor was completely refreshed at 1, 2 and 4 hours. The complete refreshing of the donor and receiver solutions was performed to minimise pH drifts that might occur due to components of the skin leaching out during the experiment. During the passive experiment, the receiver was only sampled at 3 and 6 hours. The table below describes the experiments presented in this section.

Table 4.2: Summary of the experiments on the effect of pH on the iontophoresis of NTX and BUP.

NTX in donor (mg/ml)	BUP in donor (mg/ml)	pH of donor (60 mM TrisHCl)	pH of receiver (60 mM TrisHCl)	Current intensity (current density)
0.14	1.0	4	4	0.285 mA (0.3 mA/cm^2)
0.14	1.0	5	5	
0.14	1.0	6	6	
0.14	0	6	6	
0.14	1.0	5	5	Passive

Section C: Effect of drug concentration

The side-bi-side cells used in this section allowed 0.95 cm^2 of skin to be exposed and available for drug transport. The volume of both the donor and receiver solutions was 3.5 ml. The donor solution was 0.5, 1 or 2.5 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl. pH was adjusted to 5 with NaOH or HCl. The receiver solution was 60 mM TrisHCl (pH adjusted to 5 with NaOH or HCl). A fixed current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3 times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. The donor was completely refreshed at 1, 2 and 4 hours. The table below describes the experiments presented in this section.

Table 4.3: Summary of the experiments on effect of concentration of BUP in the donor solution. *Data from section B are shown for comparison in this section and in section D.

NTX in donor as mg/ml (or molar fraction)	BUP in donor as mg/ml (or molar fraction)	Donor	Receiver	Current intensity (current density)
0.14 (0.006)	0.5 (0.016)	Tris pH 5, 60 mM	Tris pH 5, 60 mM	0.285 mA (0.3 mA/cm^2)
0.14 (0.006)*	1.0 (0.032)*			
0.14 (0.006)	2.5 (0.076)			

Section D: Effect of current density

In this section a set of side-bi-side cells were used which allowed a 'large' (3.80 cm^2) area of skin to be exposed and available for transport. The results were then compared to a 'standard' set of cells which allowed only 0.95 cm^2 of skin to be exposed (data from section B). The volume of the donor and receiver solutions in the standard cell was 3.5 ml. The volume of the donor and receiver solutions of the large cell was 8 ml. The donor solution was 1 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl. pH was adjusted to 5 with NaOH or HCl. The receiver solution was 60 mM TrisHCl (pH adjusted to 5 with NaOH or HCl). A fixed current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The donor and receiver were completely refreshed 3 times (at 1, 2 and 4 hours, and 1, 2 and 3 hours respectively). At the remaining hourly samples, 1 ml was sampled from the receiver and replenished.

Although two sizes of skin were used in this section, the same size area was tape-stripped in both cases (0.785 cm^2) so that amount of drug recovered would be directly comparable. The table below describes the experiments presented in this section.

Table 4.4: Summary of the experiments on the effect of current density.

NTX in donor (mg/ml)	BUP in donor (mg/ml)	Donor	Receiver	Current density (current intensity)	At end of 6h
0.14	1.0	60 mM Tris pH 5	60 mM Tris pH 5	'Standard' 0.3 mA/cm^2 (0.285 mA) 'Low' 0.075 mA/cm^2 (0.285 mA)	Tape-stripping

3. Results and Discussion

Section A: Feasibility study

The objective of these experiments was to determine the feasibility of iontophoresis as a technique to deliver therapeutic amounts of BUP and NTX through skin. For the initial experimental conditions of the feasibility study, a donor solution with drug concentrations close to the observed maximum aqueous solubilities was selected (1 mg/ml and 55 mg/ml for BUP and NTX respectively, at pH 6). A pH of 6 was selected as a starting point because at this pH NTX and BUP, as weak bases with pKas around 8, are both positively charged, and have acceptable aqueous solubility. Furthermore, at pH 6, EO would be expected to

contribute to overall drug flux. The results obtained can be seen in tables 4.5 and 4.6 and figures 4.6 and 4.7.

As expected, iontophoresis dramatically increased flux of both compounds compared to passive diffusion (600-fold for NTX and 40-fold for BUP). The highest iontophoretic fluxes of BUP observed here were not as high as previously reported by Fang *et al.* (2002), which was 10.1 µg/h/cm^2. However, this may be explained by their use of nude mouse skin, as rodent skin is generally considered to be more permeable than pig skin. The fluxes of NTX observed here were several fold higher than the highest NTX fluxes previously reported; Wermeling *et al. (*2008) and Ghosh *et al.* (2013) used microneedle technology and reported fluxes of 14.7 µg/h/cm^2 and 16.4 µg/h/cm^2 respectively.

Table 4.5: Iontophoretic and passive fluxes (mean ± standard deviation) of NTX and BUP across excised pig skin as a function of NTX concentration in the donor solution. The background electrolyte in all donor solutions was Tris buffer pH 6. Receiver solution was PBS pH 7.4. Current intensity was 0.38 mA over an area of 0.95 cm^2.

NTX & BUP (mg/ml)	n	NTX		BUP	
		6 h cumulative delivery (µg)	Flux at 6h (µg/h)	6 h cumulative delivery (µg)	Flux at 6h (µg/h)
55 & 1	4	1223 ± 97	272 ± 42	0.49 ± 0.29	0.22 ± 0.10
5.5 & 1	4	416 ± 169	113 ± 43	0.86 ± 0.56	0.38 ± 0.22
0.55 & 1	10	100 ± 20	24 ± 3	6.29 ± 5.25	2.04 ± 1.29
0 & 1	4	-	-	6.31 ± 2.98	1.90 ± 0.68
0.55 & 1 (passive)	4	*0.11 ± 0.13*	*0.04 ± 0.04*	*0.27 ± 0.31*	*0.05 ± 0.04*

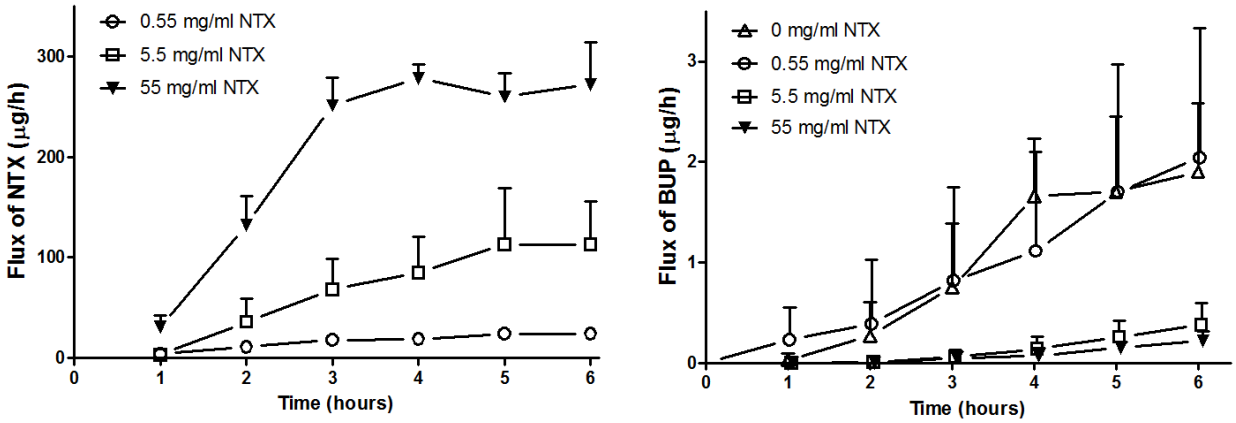

Figure 4.6: Iontophoretic flux (mean + standard deviation) of NTX (**left**) and BUP (**right**). Donor solutions were pH 6 and contained 0, 0.55, 5.5 or 55 mg/ml NTX and 1 mg/ml BUP (n = 4, 10, 4 and 4 respectively). Note the 100-fold difference in the y axes.

As expected, increasing the concentration of NTX in the donor solution increased flux of NTX and decreased flux of BUP. The flux of BUP is influenced by the amount of NTX in the system due to competition for charge-carrying between these co-ions (Mudry *et al*. 2006a and b). As the concentration of NTX in the donor solution is increased, the drug carries a larger share of the available charge in the system, it follows that another ion/s must carry less charge. Unfortunately, the amount of charge carried by the counter-ion chloride is relatively constant in this type of system. This was illustrated by Mudry (2006b) who measured the fluxes of sodium, lithium, ammonium and potassium delivered from the anode. The only counter-ion present in the cathodal compartment was chloride (present as $MgCl_2$). It was shown that altering the concentrations of lithium, ammonium and potassium in the anode compartment (while keeping the concentration of sodium fixed) altered their fluxes, but at the expense of sodium flux, not at the expense of chloride flux. When considering the data presented here, it follows that as the concentration of NTX in the donor solution increases and the flux of NTX increases, it is the flux of bulky BUP and not the flux of small mobile chloride (in the cathode-to-anode direction) or Tris+ (in the anode-to-cathode direction) that will 'lose out'.

The highest flux of BUP, and the delivery ratio of the two drugs closest to the 3:1 NTX:BUP aim determined in chapter 2, were achieved using a donor solution containing 0.55 mg/mL NTX and 1 mg/mL BUP; the fluxes were 24 and 2 µg/h at 6 hours respectively. The transport number of a drug gives a measure of its ability to compete as a charge-carrier. The transport number of BUP in the 'best' donor solution (0.55 mg/mL NTX and 1 mg/mL BUP) was 2.9 x 10^{-4} (table 4.6). This is consistent with previously reported values for transport numbers of BUP: 5 x 10^{-4} (Robson 1988, using a citrate-phosphate buffer pH 5), 9 x 10^{-4} (Bose *et al.* 2001, using a citrate buffer donor solution pH 4.0), and 10 x 10^{-4} (Fang *et al.* 2002, using a citrate-phosphate buffer pH 5). The detail provided in these three previous studies was not

sufficient to calculate the molar fraction of BUP in the donor solutions for comparison with the results presented here. The transport number of NTX in the 'best' donor was 0.0045; no comparison iontophoretic data for NTX has been published. To clarify, the vast majority of charge is carried by ions other than NTX or BUP, specifically by Tris cations in the anode-to-cathode direction, and by chloride anions in the cathode-to-anode direction.

Table 4.6: Molar fraction in the donor and transport number of NTX and BUP. All donor solutions were pH 6. Current intensity was 0.38 mA over an area of 0.95 cm^2.

NTX & BUP (mg/ml)	NTX		BUP	
	Molar fraction	Transport number	Molar fraction	Transport number
55 & 1	0.702	0.0507	0.0096	3.1×10^{-5}
5.5 & 1	0.191	0.0211	0.0259	5.3×10^{-5}
0.55 & 1	0.023	0.0045	0.0313	2.9×10^{-4}
0 & 1	n/a	n/a	0.0321	2.7×10^{-4}

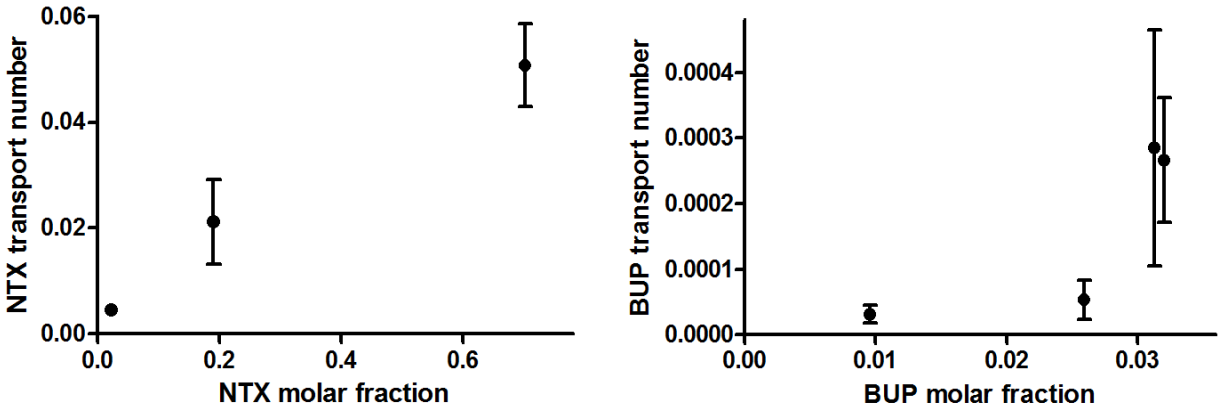

Figure 4.7: Transport number of NTX (**left;** n = 10, 4 and 4) and BUP (**right;** 4, 4, 10, 4) as a function of their molar fractions in the donor solutions. Transport number is the fraction of current that the drug carries, and is normally expected to be linearly related to molar fraction of the drug in the donor solution. All donor solutions were pH 6. Current intensity was 0.38 mA over an area of 0.95 cm^2. Values are mean ± standard deviation.

Figure 4.7 shows that for NTX, there is a linear relationship between molar fraction of the drug in the donor solution and transport number (R^2 = 0.996). BUP transport number also increases with BUP molar fraction, but not in a linear fashion (R^2 = 0.636). A non-linear

relationship between transport number and molar fraction has been observed for cations such as propranolol (Marro *et al*. 2001b) and apomorphine (van der Geest *et al.* 1997). However, the transport number normally reaches a plateau as molar fraction increases, as opposed to increasing as seen in this case. The non-linear relationship observed here might reflect the very low molar fractions used (up to only 0.03). Previous studies demonstrating a linear relationship between molar fraction on transport number have often used much higher molar fractions; for example, Mudry *et al.* (2006a) examined the behaviour of lidocaine over a range of molar fractions from 0.25 up to 1. It might be the case that the relationship between molar fraction and transport number does not follow this same behaviour when looking at changes of very low (or very high) molar fractions.

The slope of the regression of the molar fraction and transport number is a measure of the ability of the ion to compete for charge-carrying, and has been linked to aqueous mobility; a higher slope value indicates a higher efficiency and a more mobile species (Mudry *et al.* 2007). The slope for NTX was 0.075 ± 0.007 (significantly different from zero). As the relationship between molar fraction and transport number for BUP was not linear, two slope values were calculated, one for molar fractions 0.0075 to 0.0239, and one for molar fractions 0.0239 to 0.0315. The two slope values were 0.0022 ± 0.0004 and 0.0070 ± 0.002 respectively. Taking either of these values, it is clear that NTX is a far greater charge carrier than BUP. This is perhaps due to NTX's lower molecular weight and greater hydrophilicity.

In summary, iontophoresis dramatically increased flux of both compounds compared to passive diffusion. The relationship between molar fraction of the drug in the donor solution and transport number may allow the iontophoretic fluxes of NTX and BUP to be manipulated by altering their relative concentrations in the donor solution.

The target delivery rates calculated in chapter 1 were 15.2 μg/cm^2/hr for NTX and 2.2 μg/cm^2/hr for BUP. The target for NTX was far exceeded here; at 6 hours a flux of 1287 μg/cm^2/hr was observed (1223 ± 97 μg/hr across 0.95 cm^2 of skin). The target for BUP was met here; at 6 hours a flux of 2.15 μg/cm^2/hr was observed (2.04 ± 1.29 μg/hr across 0.95 cm^2 of skin). Iontophoresis may therefore be a promising route for delivering NTX and BUP through the skin.

The target fluxes for NTX and BUP predicted in chapter 1 were based on literature values of clearance and therapeutic plasma concentrations, therefore are only an approximation. Furthermore, it is unknown how well the *in vitro* iontophoretic fluxes reported here predict *in*

vivo iontophoresis fluxes. Therefore, efforts to better understand how to manipulate the relative fluxes of the two drugs were continued in subsequent sections.

Section B: Effect of pH

The effect of pH on the iontophoretic delivery of BUP and NTX was studied next. The effect of pH in an iontophoretic system can be complex due to simultaneous effects on the drug and skin charge. This is illustrated by experiments using 5-fluorouracil, a weak acid (Merino *et al*. 1999). At pH 8.5, 5-fluorouracil is largely negatively charged, and flux is predominantly in the cathode-to-anode direction, as expected as the drug is transported in this direction by ER. At pH 6, 5-fluorouracil was 99 % unionised, therefore negligible ER occurred. The contribution to total flux from EO therefore increased, and flux in the anode-to-cathode direction dominated. At pH 3, the net charge on the skin is positive, and EO is in the cathode-to-anode direction. Therefore 5-fluorouracil flux was again predominantly in the cathode-to anode direction (as at pH 8.5) but now due to EO.

A study by Lopez *et al.* (2001) using 5-aminolevulinic acid illustrates a similar point. EO (indicated by the flux of mannitol) increased dramatically from pH 4 to pH 7.4, as expected. The contribution to total flux of EO was, however, offset, by a decrease in the contribution from ER, as the fraction of 5-aminolevulinic acid in the cationic form decreased at the same time. Overall, total flux of 5-aminolevulinic acid at pH 4 and 7.4 was not different.

A pH range of 4 to 6 was chosen here. The lower limit of this range was selected because prolonged exposure to pHs lower than 4 may cause skin irritation. The upper limit of this range was selected because at pHs higher than 6, the aqueous solubility of BUP drops dramatically (table 4.7). Furthermore, although EO would be expected to increase as the pH was increased from 4 to 6, no further benefit would be expected at pHs higher than pH 6 (Marro *et al.* 2001a), probably because the skin is already maximally negatively charged at close to pH 6. Conversely, it was expected that ER contribution to total flux would be greatest at the lower pH, due to a higher ionised fraction of drug at pH 4 than at pH 6 (Siddiqui *et al*. 1989; Kalia *et al*. 2004).

Table 4.7: Percentage ionisation and aqueous solubility of NTX and BUP as a function of pH. BUP solubility values are experimental, and are taken from Robson (1988). NTX solubility values are predicted and were generated using MarvinSketch 6.3.1. The percentage of drug ionised was calculated from pKa values for NTX and BUP of 8.13 and 8.37 respectively.

	NTX		BUP	
pH	% ionised	Solubility in water (mg/ml)	% ionised	Solubility in water (mg/ml)
4	100.0	378	100.0	19.0
5	100.0	378	100.0	11.9
6	99.6	378	99.8	1.4
7.4	91.1	23.3	94.6	0.045

As well as the amount of drug that reached the receiver compartment, in this and subsequent sections, the amount of drug in the stratum corneum (the outer non-viable layer of skin) was also quantified. Measuring the concentration of drug in the skin itself is more common when delivering drugs that act locally, and not drugs that act systemically, as BUP and NTX do. These measurements were included here because there was an expectation that BUP might form a reservoir in the skin during transdermal delivery. This possibility was indicated by work by Bose *et al.* (2001), who observed a significant release of BUP from the skin upon termination of iontophoresis, though the amount of BUP in the skin was not directly measured in their study.

In order to assess the potential impact of a BUP reservoir on EO, and consequently on the fluxes of the two drugs, apparent convective solvent flow was now also measured. This was achieved by measuring the transdermal iontophoretic flux of an uncharged marker added to the donor solution. Acetaminophen (ACM), was chosen for this purpose. It has negligible passive flux, and is neutral over the pH range used here thus is not transported by ER, so is commonly used as a marker of EO (Dubey & Kalia 2014).

The objective of the experiments in this section was to determine the influence of pH on the flux of NTX and BUP, on EO, and on the amount of drug in the skin. The results are shown in tables 4.8, 4.9 and 4.10 and figures 4.8 to 4.11.

Table 4.8: Iontophoretic and passive fluxes of NTX, BUP and the marker ACM as a function of pH. Donor and receiver solution were both Tris buffer. Current intensity was 0.285 mA over an area of 0.95 cm^2. Letters in superscript indicate pairs of values significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

		NTX		BUP		ACM	
pH of donor and receiver	n	6 h cumulative delivery (µg)	Flux at 6h (µg/h)	6 h cumulative delivery (µg)	Flux at 6h (µg/h)	6 h cumulative Delivery (µg)	Flux at 6h (µg/h)
4	6	10.7 ± 2.3	3.0 ± 0.5	5.6 ± 4.5	2.6 ± 1.8	2.9 ± 1.5	0.8 ± 0.4
5	6	8.2 ± 3.1	2.8 ± 0.7	2.2 ± 2.1	1.4 ± 1.0	2.5 ± 0.9	0.8 ± 0.2
6	6	10.5 ± 1.4[a]	3.3 ± 0.3[b]	4.8 ± 2.8	2.4 ± 1.4	3.9 ± 1.1[c]	1.1 ± 0.3[d]
6 (no BUP)	4	14.1 ± 1.3[a]	4.0 ± 0.4[b]	n/a	n/a	6.0 ± 1.6[c]	1.8 ± 0.3[d]
5 (passive)	3	0	0	0	0	0	0

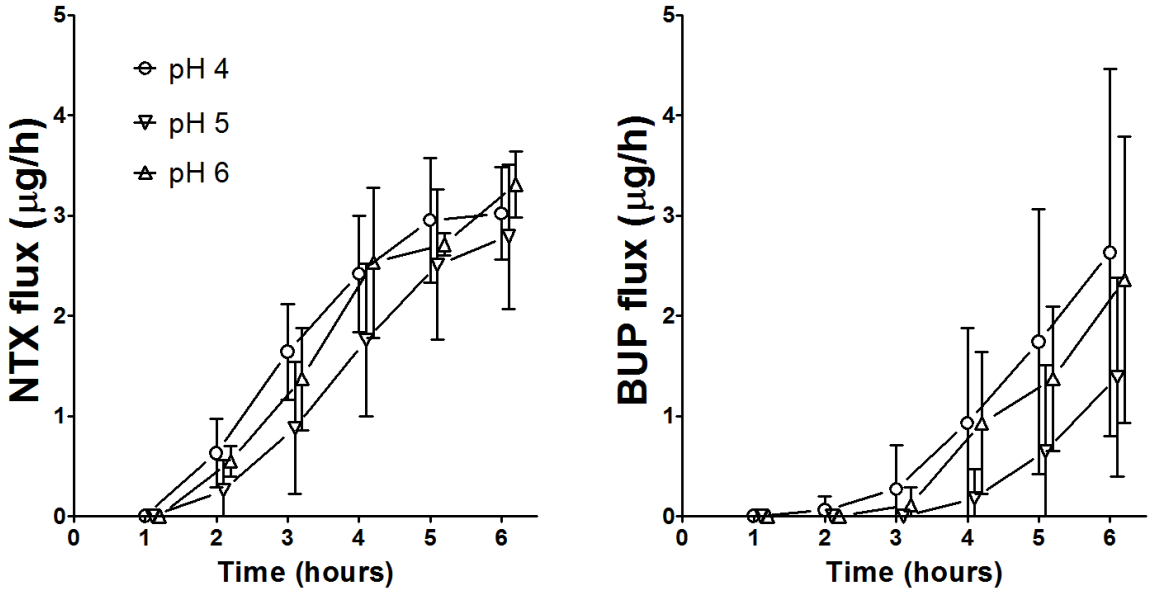

Figure 4.8: Iontophoretic fluxes of NTX (**left**) and BUP (**right**) as a function of time and pH of donor solution. All donor solutions contained 0.14 mg/ml NTX and 1.0 mg/ml BUP. pH of donor and receiver was 4, 5 or 6 (n = 6, 6 and 6). Data have been nudged to facilitate visualisation. All values are mean ± standard deviation.

The transport numbers of NTX and BUP were 0.001 and 0.0004 respectively at pH 6 (table 4.9) despite NTX being present in the donor at a concentration 7 times lower than BUP. When the transport number of the ion of interest is high, ER dominates, and the contribution of EO is small; for ions with a small transport number, EO assumes a much greater significance (Luzardo-Alvarez *et al.* 1998). It follows that the contribution of EO to total flux is less important for NTX than it is for BUP, and any change in EO will have a comparatively greater effect on the total flux of BUP. As a general rule, electrical mobility will decrease with molecular weight and as a consequence, the electroosmotic contribution should become increasingly important for larger molecules (Guy *et al*. 2000).

Table 4.9: Transport numbers of NTX and BUP as a function of pH.

	Transport number of NTX	Transport number of BUP
pH 4	7.5×10^{-4}	4.9×10^{-4}
pH 5	7.0×10^{-4}	2.6×10^{-4}
pH 6	8.2×10^{-4}	4.5×10^{-4}
pH 6 no BUP	10.0×10^{-4}	n/a

No effect of pH on flux was observed for either NTX or BUP. As mentioned, increasing pH was expected to result in:
1) a decrease in the ER contribution to the flux of NTX and BUP, as the percentage of drug present in the ionised fraction decreased (Siddiqui *et al*. 1989; Kalia *et al*. 2004) and
2) an increase in the EO contribution to the flux of NTX and BUP as the permselectivity of the skin increased (Marro *et al.* 2001a).

The lack of effect of pH on flux observed here cannot be explained by these two (opposing) factors cancelling each other out, as it had been for the 5-aminolevulinic acid work (Lopez *et al.* 2001). Lopez *et al.* saw a clear increase in EO with increasing pH, as indicated by the flux of the marker mannitol. However, that was not the case for the data presented here, and surprisingly, no effect of pH on the flux of ACM was observed. To explain why no influence of pH on EO was observed, it is useful to consider the amount of BUP recovered from the stratum corneum.

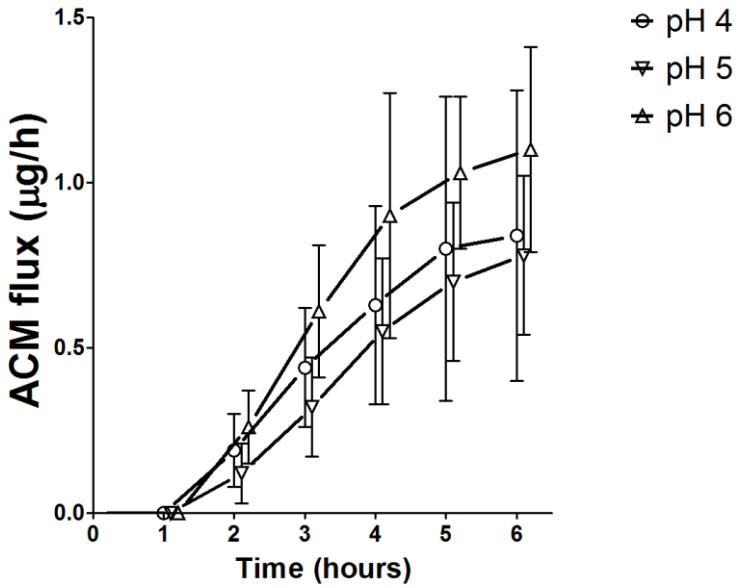

Figure 4.9: Flux (mean ± standard deviation) of ACM as a function of pH. ACM is a marker of EO convective flow. All donor solutions contained 0.14 mg/ml NTX and 1.0 mg/ml BUP. pH of donor and receiver was 4, 5 or 6 (n = 6, 6 and 6). Data have been nudged to facilitate visualisation.

NTX did not accumulate in the stratum corneum (NTX was not quantifiable beyond tape 1). In contrast, the amount of BUP recovered from the stratum corneum was large compared to the amount that reached the receiver compartment. For example, at pH 6, the total amount of BUP that crossed into the receiver solution was 4.8 ± 2.8 µg and the amount of BUP recovered from the stratum corneum was 151 ± 137 µg (192 ± 175 µg/cm^2). To put the amount of BUP in the stratum corneum observed here in context, Curdy *et al*. (2001) recovered 49 µg/cm^2 of piroxicam from the stratum corneum following cathodal iontophoretic delivery *in vivo* in man. Jadoul *et al.* (1995) reported recovering 0.1 µg/cm^2 fentanyl, and 0.25 µg/cm^2 thyrotropin releasing hormone, from the stratum corneum following anodal iontophoretic delivery in rat skin *in vitro*.

The accumulation of BUP in the stratum corneum was pH-dependent (table 4.10). The mass of BUP recovered from the stratum corneum was 5 times higher at pH 6 than at pH 4 (table 4.10). The depths of stratum corneum reached for pH 4, 5 and 6 were 4.8 ± 1.8 µm, 5.2 ± 2.7 µm, and 7.2 ± 2.2 µm. These depths are not statistically different. Nevertheless, in case the depth sampled influenced the amount of BUP recovered in the stratum corneum, the results were also reported as concentration of drug in the stratum corneum; the concentration of BUP in the stratum corneum was three times higher at pH 6 than at pH 4. It has been previously reported that passive BUP partitioning into isolated human stratum corneum was pH-dependent (Robson 1988). For example, the log of the stratum corneum–aqueous buffer distribution ratio of BUP (as the hydrochloride) increased from 0.5 at pH 4 to 2.5 at pH 7.4.

Table 4.10: Effect of pH on accumulation of BUP in the stratum corneum (NTX not quantifiable in stratum corneum beyond tape 1). Mean ± standard deviation.

pH of donor and receiver	BUP in tapes (µg)	Stratum corneum removed (µg)	Concentration of BUP in stratum corneum (µg/mg)
4	15.3 ± 5.7	376 ± 144	51.3 ± 34.0
5	23.3 ± 5.7	408 ± 214	81.2 ± 62.9
6	84.5 ± 79.1	569 ± 173	151.0 ± 136.7
5 (passive)	3.2 ± 1.3	772 ± 203	4.1 ± 0.6

As each tape was extracted and analysed separately, the distribution profile of BUP in the stratum corneum can be plotted (figure 4.10). The profiles show a typical decrease in drug in the stratum corneum with increasing depth. The average depth of stratum corneum sampled was 6 µm, out of an available total thickness of porcine stratum corneum of around 10 µm (Herkenne *et al.* 2006).

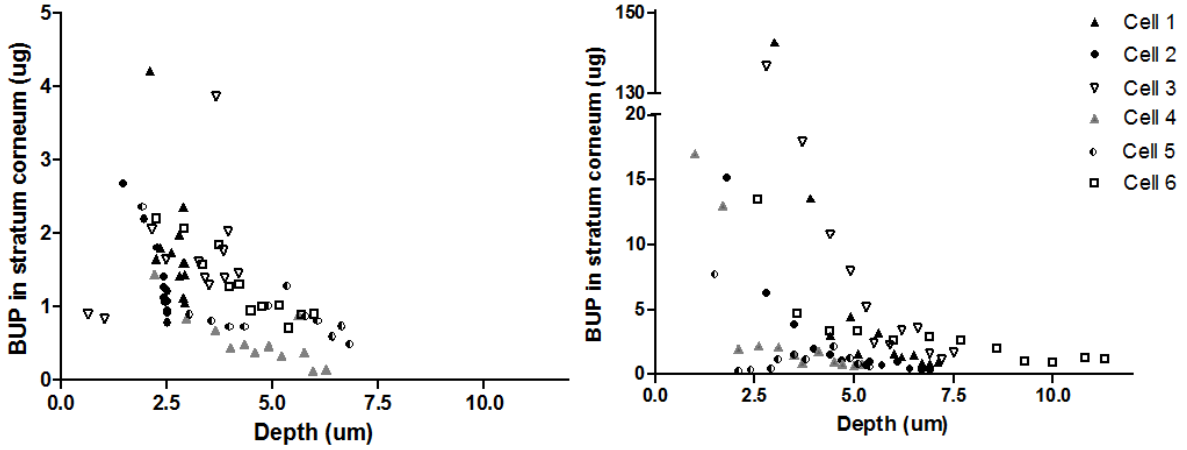

Figure 4.10: Amount of BUP in stratum corneum as a function of depth, following 6 hours of iontophoresis. All donor solutions contained 0.14 mg/ml NTX and 1.0 mg/ml BUP. Donor solution and receptor solution were pH 4 (**left**) or pH 6 (**right**), n = 6 and 6.

Tape-stripping following the passive experiment yielded little BUP (3 ± 1 µg). Importantly, this low amount recovered for the passive experiment indicates that BUP recovered from the stratum corneum after iontophoresis was not simply residual donor solution on the surface of

the skin. NTX in the stratum corneum was below limit of quantification following the passive experiment.

The difference between the amounts of BUP and NTX in the stratum corneum may reflect the fact that NTX was present in the donor at a concentration 7 times lower than BUP. Alternatively, it may be because NTX is less lipophilic than BUP therefore less attracted to the lipid environment of the stratum corneum. Delgado-Charro and Guy (1994) commented that as well as an electrostatic attraction between a positively charged drug and the negatively charged sites in the skin, some lipophilicity of the drug also appears necessary for accumulation to occur. NTX and BUP are both similarly positively charged at this pH, but only BUP accumulates in the skin. Lastly, it is possible that the difference in recovery of NTX and BUP from the stratum corneum is a reflection of different pathways taken through the skin by these two drugs. Curdy *et al.* (2001) suggested that permeants which travel via the follicular route and move faster through the stratum corneum would be expected to found in the stratum corneum at lower concentrations. Correspondingly, Curdy *et al.* (2001) inferred that if a permeant is found in the stratum corneum it must be at least partly transported via the intercellular route.

Influence of accumulated BUP on EO and on flux of NTX

An explanation for the surprising absence of effect of pH on EO is now offered. It was hypothesised that at pH 6, the skin depot of BUP was neutralising negative charges on the skin and suppressing EO. Suppression of EO by accumulation of a cation in the skin has been previously reported for the peptide nafarelin (Delgado-Charro *et al*. 1995; Santi & Guy 1996a). To test whether BUP was causing suppression of EO, iontophoresis was performed (at pH 6 only) in the absence of BUP. Note that removal of BUP from the donor solution increased the molar fraction of NTX by only 3%, therefore, any change in flux of NTX associated with ion competition would not be expected to be measurable. The results are shown in figure 4.11.

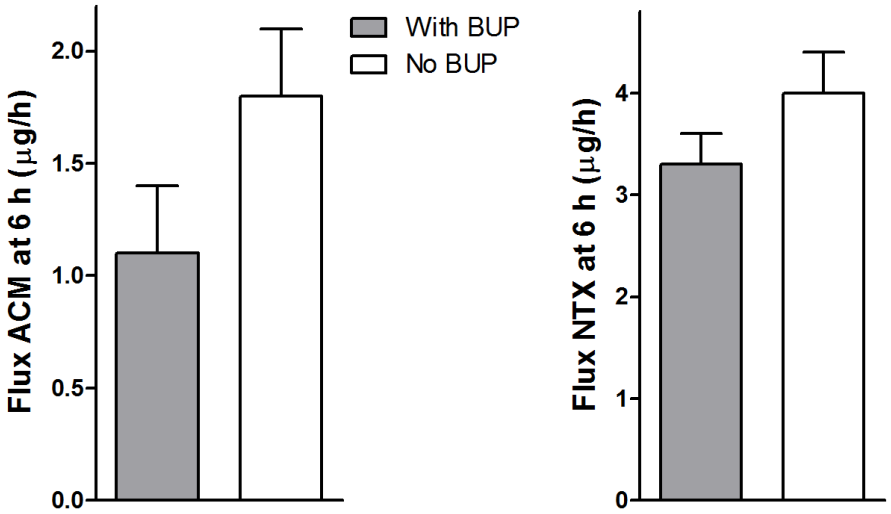

Figure 4.11: Flux of ACM (**left**) and flux of NTX (**right**) in the absence and presence of 1 mg/ml BUP in the donor. Both donor solutions contained 0.14 mg/ml NTX and the pH of the donor solution and the receptor solution was pH 6 (n = 6 and 6). Data shown are mean + standard deviation.

Significantly higher fluxes of ACM were indeed observed in the absence of BUP. This observation supports the hypothesis that BUP suppresses EO. At pH 6, the observed accumulation of BUP in the skin may be cancelling the negative charges in the skin that normally generate EO flow.

Significantly higher fluxes of NTX were also observed in the absence of BUP. The increase in NTX flux (21 %) is smaller than the increase in ACM flux (64 %). This is because, unlike ACM, NTX total flux is composed of contributions from ER as well as from EO.

Having demonstrated that BUP inhibits EO, and thereby inhibits flux of NTX, it was inferred that BUP also inhibits its own flux. Furthermore, it is expected that any reduction in EO will have a greater impact on the flux of BUP than on the flux of NTX. That is because BUP is less efficient at competing to carry charge than NTX (as indicated by lower transport numbers than NTX), therefore the relative contribution of EO to total flux of BUP will be greater than the relative contribution of EO to total flux of NTX (Luzardo-Alvarez *et al.* 1998, Guy *et al*. 2000).

To the author's knowledge, it has not been previously reported that during simultaneous iontophoretic delivery of two drugs, one drug could alter the flux of the other drug, by a mechanism not related to ion competition. Thysman *et al.* (1994a) studied the iontophoretic flux of two drugs delivered from the anode in succession (calcitonin for 1 hour followed by metroprolol for 6 hours). It was shown that delivery of calcitonin altered the skin in a way that reduced the subsequent flux of metroprolol. According to Thysman, this effect might be

explained by physical blocking of ‘skin channels’ or ‘pores’ by calcitonin, but the authors did not explore changes in EO.

In summary, BUP accumulates in the stratum corneum in a pH-dependent fashion, reducing EO, probably by cancelling the net negative charge of the skin. Consequently, BUP limits its own flux, as well as negatively impacting on the flux of NTX.

Section C: Effect of drug concentration

The effect of drug donor concentration during iontophoresis is usually predictable, with flux proportional to molar fraction of drug in the donor (Phipps & Gyory 1992; Marro *et al.* 2001c; Kalia *et al.* 2004). However, this is not always the case (Hoogstraate *et al.* 1994; Merino *et al.* 2008; Dubey & Kalia 2014). In section A, the concentration of BUP in the donor solution was constant, and the concentration of NTX in the donor solution was varied. In this section, the concentration of NTX in the donor solution was kept constant and the concentration of BUP in the donor solution was varied.

The objective of these experiments was to determine the influence of BUP concentration in the donor on flux of NTX and BUP, on EO and on amount of drug in the stratum corneum and the viable tissue. Tables 4.11 and 4.12 and figures 4.12 and 4.13 show the results.

Despite a five-fold increase in the concentration (and molar fraction) of BUP in the donor solution, no increase in the flux of BUP was observed. This can be explained by the auto-inhibition of BUP demonstrated in the previous section.

Table 4.11: Iontophoretic fluxes of BUP, NTX and ACM as a function of different concentrations of BUP in the donor. The background electrolyte of the donor and receiver solutions was Tris buffer pH 5. Current intensity was 0.285 mA over an area of 0.95 cm^2. Mean ± standard deviation. Letters in superscript indicate pairs of values are significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

Donor (mg/ml)			BUP	NTX	ACM
NTX	BUP	n	Flux at 6 h (µg/h)	Flux at 6 h (µg/h)	Flux at 6 h (µg/h)
0.14	0.5	4	3.5 ± 1.7	3.6 ± 0.4[a]	0.9 ± 0.1[b]
0.14	1.0	6	1.4 ± 1.0	2.8 ± 0.7	0.8 ± 0.2[c]
0.14	2.5	4	3.9 ± 1.9	2.3 ± 0.3[a]	0.4 ± 0.0[b,c]

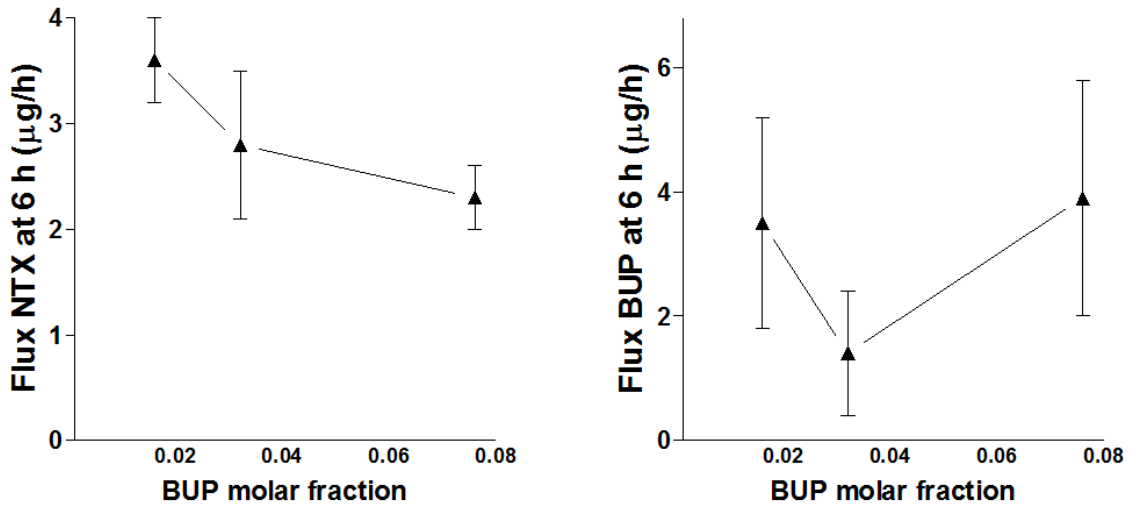

Figure 4.12: Flux of NTX (**left**) and BUP (**right**) as a function of BUP molar fraction in the donor. The background electrolyte in both the donor solution and the receptor solution was 60 mM Tris pH 5. Donor solutions contained 0.14 mg/ml NTX, either 0.5, 1.0 or 2.5 mg/ml BUP (n = 4, 6 and 4 respectively), and 0.5 mg/ml ACM. Mean ± standard deviation.

As the concentration of BUP in the donor solution was increased, the amount of BUP accumulated in the skin increased (figure 4.13, right panel). In this section, as well as the amount of BUP in the stratum corneum, the amount of drug in the underlying viable skin tissue was quantified. A similar amount of BUP was recovered from the viable tissue and the stratum corneum, though obviously the concentration of BUP in the viable tissue was much lower, as the thickness of the stratum corneum is ~10 µm and the thickness of the viable tissue was ~750 µm. The lower concentration of BUP in the viable tissue most likely reflects slow partitioning of this lipophilic drug from the stratum corneum into a comparatively

aqueous environment. Interestingly, and as already seen in section B, the amount of BUP in the skin is not a good predictor of BUP transdermal flux. NTX was quantifiable in the viable skin tissue, but as expected, no effect of concentration of BUP on amount of NTX in the viable tissue was observed.

In agreement with the results in the previous section, increasing the concentration of BUP in the donor solution decreased the flux of NTX. The decrease in NTX flux cannot be explained by simple ion competition; the molar fraction of NTX in the donor solution was only decreased by 6 % when the concentration of BUP was increased from 0.5 mg/ml to 2.5 mg/ml and so cannot account for the observed 57 % decrease in NTX flux. Instead, the decrease in NTX can be explained by an effect on EO.

The flux of ACM (the marker of EO) decreased with increasing concentration of BUP in the donor; EO decreased by 60% when the concentration of BUP in the donor was increased from 0.5 mg/ml to 2.5 mg/ml (figure 4.13, left panel). This is not an effect of ionic strength; the background electrolyte in the donor is 60 mM, increasing the concentration of BUP from 0.5 to 2.5 mg/ml is only an increase of 4 mM. Rather, the effect of BUP concentration on EO is most likely the result of the accumulation of BUP in the skin, cancelling the negative charges of the skin.

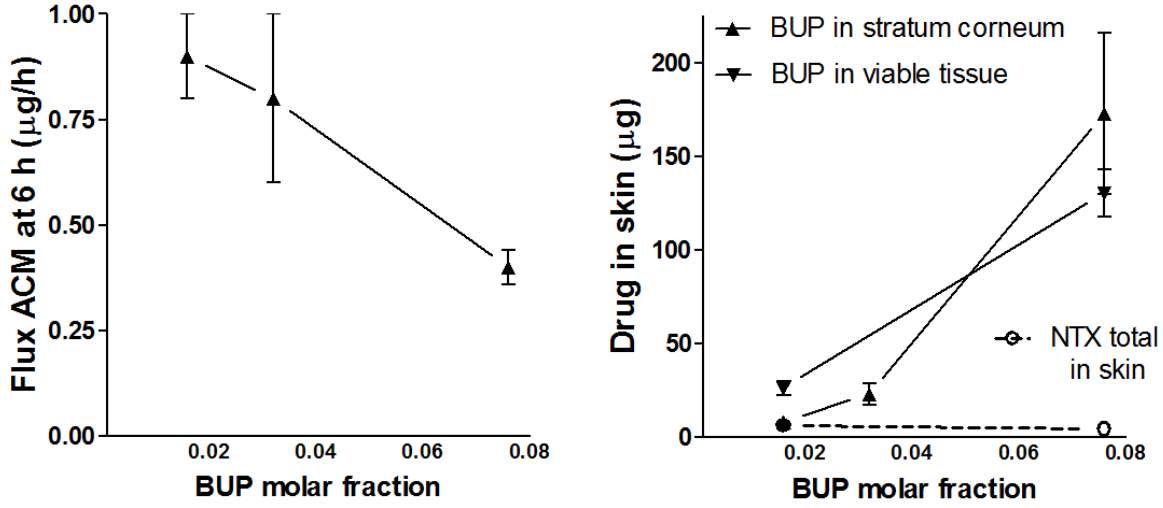

Figure 4.13: Flux of ACM (**left**), and amount of BUP and NTX in the skin (**right**) as a function of BUP molar fraction in the donor. The background electrolyte in both the donor solution and the receptor solution was 60 mM Tris pH 5. Donor solutions contained 0.14 mg/ml NTX, either 0.5, 1.0 or 2.5 mg/ml BUP (n = 4, 6 and 4 respectively), and 0.5 mg/ml ACM. Mean ± standard deviation.

Table 4.12: Amount of BUP and NTX (mean ± standard deviation) in the skin as a function of BUP donor concentration. Letters in superscript indicate pairs of values significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

Donor (mg/ml)		BUP				NTX
NTX	BUP	Drug in stratum corneum (µg)	Stratum corneum removed (µg)	Concentration of BUP in stratum corneum (µg/mg)	Drug in viable epidermis (µg)	Drug in viable epidermis (µg)
0.14	0.5	8.8 ± 1.5[a]	545 ± 63	16.1 ± 2.5[d]	27.9 ± 3.9[f]	6.6 ± 1.4
0.14	1.0	23.3 ± 5.7[b]	408 ± 214[c]	81.2 ± 62.9[e]	-	-
0.14	2.5	173 ± 43[a,b]	748 ± 105[c]	228.1 ± 28.6[d,e]	130.6 ± 12.7[f]	4.8 ± 0.9

In summary, a clear auto-inhibition of BUP flux was seen: despite a 5-fold increase in BUP donor concentration, no increase in flux of BUP was observed. In other words, the effect of increasing BUP molar fraction was counteracted by a decreasing contribution of EO.

In an iontophoretic system that behaves 'conventionally', flux can be increased by manipulating the molar fraction of drug in the donor. This can be done either by increasing the concentration of the drug in the donor, or by decreasing the concentration of other ions present in the buffer. It was demonstrated in this section that this first option did not result in increased flux of BUP. The second option of reducing the concentration of the Tris buffer in the donor solution was not pursued, for the following reasons. Firstly, a minimal concentration of chloride ions is required for the electrochemistry at the anode, and secondly, reducing the concentration of the buffer would reduce the buffer capacity. Therefore, in the next section, the less well explored strategy of manipulating current density was considered.

Section D: Effect of current density

The effect of altering current density, while fixing current intensity, has been investigated by Lopez-Castellano *et al.* (1999) using propranolol, a lipophilic cation that inhibits its own flux in a concentration-dependent manner (Hirvonen & Guy 1997). Although the amount of drug

delivered during iontophoresis is normally linearly proportional to current intensity (Burnette & Ongpipattanakul 1987; Phipps *et al*. 1989; Pikal 2001), Lopez-Castellano *et al.* hypothesised that if the drug was spread out over a larger area it might have less impact on the EO contribution to iontophoretic flux. It was hoped here that this same strategy might result in a lower BUP depot per unit area of skin and therefore higher EO and, potentially, higher BUP flux.

The objective of these experiments was to determine the influence of current density on flux of NTX and BUP, on EO, and on the amount of drug in the skin. Experiments were therefore here carried out in which the current intensity was fixed but the area of exposed skin was increased, thus the current density was decreased. Tables 4.13 and 4.14 and figures 4.14 and 4.15 show the results.

Table 4.13: Iontophoretic fluxes of NTX, BUP and ACM under 'standard' or 'low' current density conditions. Current intensity was 0.285 mA over an area of 0.95 cm^2 or 3.80 cm^2. The background electrolyte for the donor and receiver solutions was Tris buffer pH 5.Mean ± standard deviation. Letters in superscript indicate pairs of values significantly different (t-test, $p < 0.05$).

		NTX		BUP		ACM	
Current density (mA/cm^2)	n	6 h cumulative delivery (µg)	Flux at 6h (µg/h)	6 h cumulative delivery (µg)	Flux at 6h (µg/h)	6 h cumulative delivery (µg)	Flux at 6h (µg/h)
Standard (0.3)	6	8.2 ± 3.1	2.8 ± 0.7	2.2 ± 2.1	1.4 ± 1.0	2.5 ± 0.9	0.8 ± 0.2[a]
Low (0.075)	4	7.4 ± 2.8	2.5 ± 0.8	3.7 ± 2.2	1.7 ± 0.7	4.0 ± 1.0	1.2 ± 0.3[a]

It was hypothesised that a lower current density might result in lower accumulation of BUP in the skin which in turn might result in less inhibition of EO by BUP. Indeed, the flux of ACM was significantly higher at the lower current density.

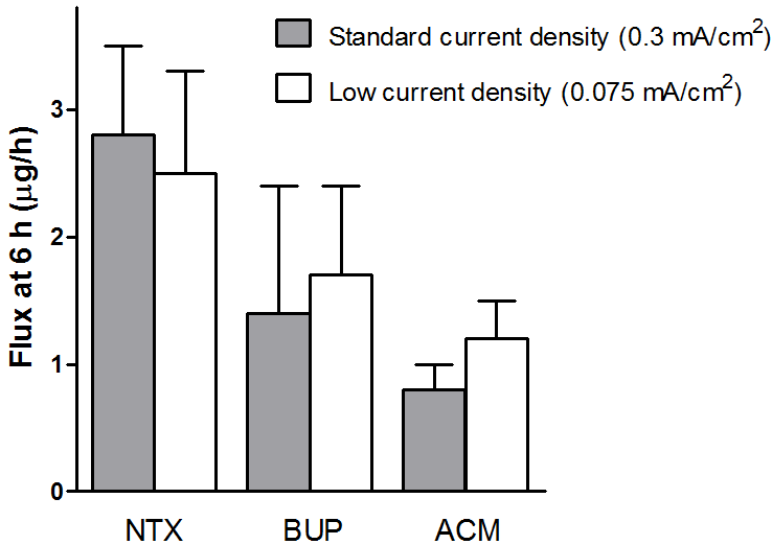

Figure 4.14: Iontophoretic fluxes of NTX, BUP and ACM as a function of current density. Both the donor solution and the receiver solution were pH 5. All donor solutions contained 0.14 mg/ml NTX and 1.0 mg/ml BUP (n = 6 and 4 for standard and low current density). Values are mean + standard deviation.

However, the difference in apparent EO observed did not translate into a statistically different flux of BUP, presumably because BUP flux is the sum of contributions from both ER and EO. Similarly, NTX was not influenced by the change in apparent EO. Lowering current density even further (whilst fixing current intensity) might give rise to higher fluxes of drug. However, considering a 4-fold decrease in current density only resulted in a ~ 50 % boost to EO such as strategy would require very large patch sizes in patients, rendering this approach impractical.

Table 4.14: Amount of BUP in the stratum corneum following iontophoresis using standard (0.3 mA/cm^2) or low (0.075 mA/cm^2) current density. The area tape stripped was 0.785 cm^2 in both cases. NTX not quantifiable past tape 1. Mean ± standard deviation. Letters in superscript indicate pairs of values are significantly different (t-test, $p < 0.05$).

Current density (mA/cm^2)	Amount of BUP in the stratum corneum (µg)	Stratum corneum removed (µg)	Concentration of BUP in stratum corneum (µg/mg)
Standard (0.3)	23.3 ± 5.7	408 ± 214[a]	81.2 ± 62.9
Low (0.075)	27.4 ± 39.5	787 ± 46[a]	34.3 ± 48.8

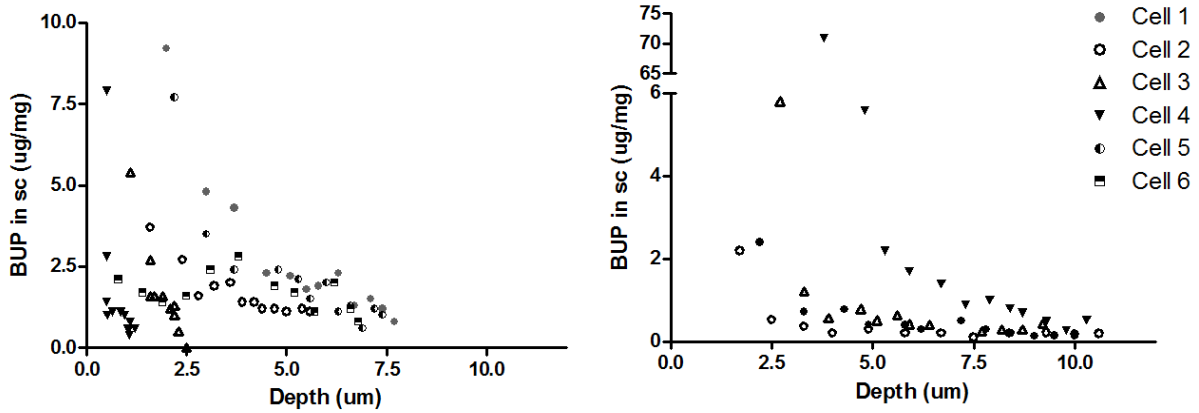

Figure 4.15: Distribution profiles of BUP in the stratum corneum following iontophoresis using standard (0.3 mA/cm^2; **left panel**) or low (0.075 mA/cm^2; **right panel**) current density. Both the donor solution and the receiver solution were pH 5. All donor solutions contained 0.14 mg/ml NTX and 1.0 mg/ml BUP (n = 6 and 4 for standard and low current density).

The explanation for the higher flux of ACM observed at the lower current density is not obvious. It had been hypothesised that current density would influence amount of BUP in the skin, which would in turn influence inhibition of EO. However, no significant effect of current density on the amount recovered nor concentration of BUP in the stratum corneum was observed (table 4.14 and figure 4.15). The explanation for the higher flux of ACM observed at the lower current density may lie in differences in the amount of BUP in the underlying viable tissue, which was not assayed here.

Although lowering current density was not successful at reducing the concentration of BUP in the stratum corneum, or at boosting BUP flux, these data do show that fluxes of BUP and NTX are determined by current intensity (over the range of current density investigated here), this would at least allow for titration of dose. This was as expected; even compounds with documented 'anomalous' behaviour, like nafarelin and ropinirole, obey this rule (Delgado-Charro *et al.* 1995; Luzardo-Alvarez *et al.* 2001).

4. Conclusions of the chapter

The transdermal flux of both drugs was dramatically increased by iontophoresis compared to the passive control. Varying the concentration of NTX in the donor solution (in the range 0.55 to 55 mg/ml) when the concentration of BUP was fixed resulted in a predictable change in the flux of NTX, as explained by ion competition. The observed flux of NTX was far higher than the predicted target, whilst the flux of BUP met the predicted target. Further experiments therefore used lower donor concentrations of NTX.

During iontophoresis a skin depot of BUP was formed. The magnitude of the reservoir was proportional to the concentration of BUP in the donor and was greater at pH 6 than at pH 4. The mechanism of formation of the depot cannot be known from these data, but is likely a consequence of both an electrostatic attraction between BUP and the skin, and the low partitioning of lipophilic BUP out of the stratum corneum into the relatively aqueous environment of the viable tissue beneath (Riviere & Heit 1997; Hirvonen & Guy 1997). The BUP depot was associated with a decrease in anode-to-cathode EO, presumably by neutralising negative charges on the skin. Interestingly, this effect was accompanied not only by significant auto-inhibition of BUP flux but also by a decreased NTX flux.

This anomalous behaviour complicates optimisation of this iontophoretic drug combination. A less common strategy of reducing current density was therefore investigated, and though a significant effect on apparent EO was observed, this approach was unsuccessful at reducing the depot or at increasing the flux of BUP.

The work presented in this chapter demonstrates that the simultaneous iontophoresis of two cations may result in complicated transport interactions beyond those expected through transport number competition. Nevertheless, iontophoresis appears to be a promising approach for delivering NTX and BUP through the skin.

For the majority of the experiments performed in this chapter the receiver solutions used were matched to the buffers used in the donor solutions. In chapter 5, further characterisation and optimisation of the system was carried out using a receiver solution more comparable to the *in vivo* situation.

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Chapter 5: Characterisation of iontophoretic transdermal delivery of buprenorphine and naltrexone *in vitro* using a physiologically relevant receiver solution

1. Introduction

In chapter 4 it was established that both NTX and BUP could be delivered by transdermal iontophoresis. However, delivery of BUP was significantly less efficient than delivery of NTX. It was established that BUP accumulates in the skin, and by its influence on EO, reduces its own flux, as well as that of NTX. Therefore, the aim of this chapter was to further understand the anomalous iontophoretic behaviour of BUP.

Furthermore, in this chapter a receiver solution of a physiological pH was adopted, in order to closer mimic the *in vivo* situation. In chapter 4, the importance of pH in an iontophoretic system was described, and it was stated that pH affects various factors simultaneously. For example, the pH of the donor solution can change the percentage of the drug which is ionised, which modifies the aqueous solubility. The percentage of drug which is ionised also influences the total drug flux, because obviously only ionised drug is transported by electrorepulsion (ER) (Siddiqui *et al.* 1989). In the introduction of this chapter, the specific importance of the composition of the *receiver solution*, including the effect of pH, will be discussed. The accumulation of drug in the skin, current profile effects and lastly molar fraction effects are also briefly discussed.

Influence of receiver solution on iontophoresis

The composition of the receiver solution can influence the iontophoretic flux of a drug, via the separate mechanisms of ER and electroosmosis (EO). The effect on ER will be discussed first. Mudry *et al*. (2006a) demonstrated that the flux of lithium from the anode was influenced by the anion (chloride, acetate, or gluconate) used in the receiver solution. The flux of lithium was highest when the receiver solution contained gluconate, and lowest when the receiver solution contained chloride. This observation can be explained by the difference in the aqueous mobility of the three anions, which are 8.1×10^{-4}, 4.2×10^{-4} and 2.5×10^{-4} $cm^2/s.V$ for chloride, acetate and gluconate respectively. There is competition for charge-carrying between lithium in the donor (transported in the anode-to-cathode direction), and the anion in the receiver solution (transported in the cathode-to-anode direction); chloride is better at competing with lithium for charge-carrying than gluconate is.

There are very few drug delivery studies which specifically examine the effect of receiver solution composition on EO. However, drug extraction studies offer useful insight into the

influence on EO of the composition of the subdermal electrolyte solution. Transdermal extraction of lithium (Leboulanger *et al.* 2004), phenylalanine (Merino *et al.* 1999) and glucose (Tamada & Comyns 2005) has been investigated for therapeutic monitoring purposes. The compounds are extracted using iontophoresis; as the aim is to transport the permeant out of the body instead of into the body, the term 'reverse iontophoresis' is commonly used. In the case of glucose, which is neutral, transport out of the skin into a collection chamber is effectively driven exclusively by EO. Several studies have therefore been carried out with the objective of selecting optimal electrolyte solutions for collection chambers to maximise extraction efficiencies. These reverse iontophoresis experiments showed that the following factors influence EO:

1. *Type of ion*. Tamada & Comyns (2005) measured iontophoretic extraction of glucose (an indicator of EO) *in vivo* in man, and showed that the extraction of glucose was more than twice as efficient into a collection chamber containing citrate buffer compared to into a collection chamber containing bicarbonate buffer (both solutions were pH 6.5 and were closely matched in ionic strength). No clear explanation of this effect of buffer type on EO has yet been proven, but it is hypothesised that different buffer molecules have a different ability to shield the skin's negative charges (Santi & Guy 1996a).

2. *Ionic strength*. Tamada & Comyns (2005) observed that lower ionic strengths in the collection chamber resulted in higher flux of glucose. Again, this effect may be related to the shielding of the negative charges in the skin, which would be expected to increase with buffer concentration. An inverse relationship between ionic strength and EO was also observed by Santi & Guy (1996b) who measured outward flux of mannitol (another marker of EO) *in vitro*.

3. *pH*. Lastly, Tamada & Comyns (2005) reported that higher pH in the collection chamber (pH 7.5 versus pH 6.5, and pH 6.5 versus 4.5) resulted in greater flux of glucose. The finding that higher pH in the receiver results in greater anode-to-cathode EO is supported by work from Kim *et al.* (1993). Several other researchers have shown in 'symmetrical' set-ups (i.e., the donor solution and receiver solution were prepared using identical background electrolyte) that higher pH results in greater EO (Luzardo-Alvarez *et al.* 1998; Marro *et al.* 2001a); Kim *et al.* however, demonstrated that the same effect could be observed by changing the pH of only the receiver compartment. Keeping all other parameters fixed, and using a donor solution of pH 4, the flux of mannitol from the anode was observed to be more than three times greater when a receiver solution of pH 7.4 was used compared to a receiver solution of pH 3.9.

Additionally, the choice of receiver solution may influence the chemical and physical stability of a drug during an experiment. For example, Delgado-Charro & Guy (1995) showed an apparent increase in the rate of degradation of nafarelin (a peptide) during the experiment when the pH of the receiver was increased from pH 5 to pH 6 or 7. Lastly, experimental artefacts such as loss of drug from the receiver solution due to adsorption to the glass surfaces of the apparatus can also be effected by choice of receiver solution.

Skin accumulation

In chapter 4, it was observed that BUP accumulates in the stratum corneum and viable tissue during iontophoresis. Some accumulation of drug in the stratum corneum is relatively common during passive delivery and is particularly well documented for topical steroids (Surber *et al.* 1990; Roberts *et al.* 2004). The accumulation of BUP in the skin is important for the following reasons. If BUP moved into the skin and remained there it would not reach the subdermal compartment (*in vivo*) to become bioavailable. Alternatively, BUP in the skin might be released into the systemic circulation after removal of a patch by a patient; this effect has been documented for fentanyl patches (Grond *et al*. 2000). A key principle of this combination product is that BUP, an opioid agonist, should not be delivered without the antagonist NTX (NTX does not form a reservoir in the skin). Lastly, as shown in section B and C of the previous chapter, the BUP depot inhibits EO and consequently has a negative impact on flux.

Accumulation of a cationic drug in the skin can mean that the conventional linear relationship between molar fraction and flux (Phipps & Gyory 1992) is not observed. For example, Luzardo-Alvarez *et al*. (2001) performed iontophoresis of ropinirole and found that increasing the concentration of ropinirole in the donor such that molar fraction was increased more than four-fold did not result in a significant increase in the flux of ropinirole. This observation was explained by a reduced contribution from EO (as indicated by mannitol flux) as the concentration of ropinirole in the donor solution was increased. This effect has been termed ‘auto-inhibition’.

Similar behaviour has been documented for peptides (Hoogstraate *et al*. 1994; Delgado-Charro & Guy 1994; Delgado-Charro & Guy 1995; Delgado-Charro *et al*. 1995; Rodriguez-Bayon & Guy 1996; Schuetz *et al.* 2005; Cazares-Delgadillo *et al*. 2007; Dubey & Kalia 2014) as well as small drug molecules (table 5.1). BUP is within the same range of lipophilicity as the other drugs known to auto-inhibit (BUP logP 2.63). That does not mean that BUP is ‘too lipophilic’ for iontophoresis; lidocaine hydrochloride (logP 2.3) and fentanyl

hydrochloride (logP of 3.8) are also within this range, and both have been formulated into commercial iontophoresis products. However, the duration of iontophoresis for both lidocaine and fentanyl is relatively short (less than 30 minutes per session).

Table 5.1: Small drug molecules observed to inhibit their own flux during transdermal iontophoresis, shown with their molecular weights and logP values.

Compound	Authors and year	Molecular weight	logP
Propranolol	Hirvonen & Guy 1997; Marro *et al.* 2001b	259	3.21
Timolol	Hirvonen & Guy 1997	316	1.91
Metoprolol	Hirvonen & Guy 1997	272	1.88
Ropinirole	Luzardo-Alvarez *et al.* 2001	260	2.70
Quinine	Marro *et al*. 2001b	324	3.44
Nortriptyline hydrochloride	Merino *et al*. 2008	298	4.54

Accumulation of drug in the skin has been attributed to electrostatic attraction between the skin and the drug (all the compounds in table 5.1 are delivered as cations), and to a certain level of lipophilicity of the drug (Hirvonen & Guy 1997; Raiman *et al.* 2003). Furthermore, as a drug moves across the skin, *in vitro* or *in vivo*, it may encounter a pH gradient. Therefore its ionisation and water solubility may change, resulting in accumulation in the skin (Thysman *et al*. 1994a). *In vivo*, the pH of the skin changes from about 4.2 at the skin surface to pH 7.3 in the aqueous viable tissue (Sage 1997).

Current profile effects

Contribution to total flux from ER can be optimised by increasing the transport number, for example by manipulation of the drug molar fraction of the donor solution. Contribution to total flux from EO can be optimised by increasing convective solvent flow, for example by manipulation of the pH. After exploring these options in this and the previous chapter, the current profile, which can influence both ER and EO, was here considered.

During an iontophoresis session the current can be (deliberately) turned off for short periods. For example, current could be switched off for 10 minutes out of every hour. This will be

referred to as 'pulsatile iontophoresis'. The original rationale for pulsatile iontophoresis was to counter iontophoresis-induced skin irritation, by allowing depolarisation of the skin (Okabe *et al.* 1986) though there is not clear published evidence of such an effect. Later, it was suggested that the depolarisation associated with an off period might result in release of drug accumulated in the skin (Yoshida & Roberts 1992; Thysman *et al.* 1994b). Lastly, pulsatile iontophoresis has been used to deliver bursts of drug to mimic the natural cycles in blood concentration of some hormones. Some previous studies employing pulsatile iontophoresis are shown in table 5.2.

Table 5.2: Some previous studies which have performed pulsatile iontophoresis, meaning an 'off' period was included. Studies where the on/off period was on a Hertz scale are not described.

Authors and year	Details	Rationale	Timings	Outcome
Miller *et al.* 1989	Anodal delivery of gonadotropin-releasing hormone. Hairless mouse, AgCl electrodes	To mimic natural pulsatile pattern of plasma levels	On and off every 10 or 20 mins for 5 h	Clear pulsatile delivery, no delivery during off period. No comparable control available.
Knoblauch & Moll 1993	Anodal delivery of buserelin. Isolated human stratum corneum, Pt electrodes	To mimic natural pulsatile pattern of plasma levels	5 min on, 55 min off, for 6 h	No comparison control vs. pulsatile. Cumulative delivery increased with current duration, though not linearly.
Delgado-Charro & Guy 1995	Anodal delivery of nafarelin. Hairless mouse skin, AgCl electrodes	Release of drug reservoir from skin	6 h on, 6 h off, for 24 h	Cumulative drug delivered $\propto$ current passed
Santi & Guy 1996b	Anodal and cathodal extraction of mannitol. Hairless mouse skin, AgCl electrodes	1. Off period is useful for sample processing 2. Reduce iontophoresis-induced inflammation	15 mins on, 5 mins off, for 2 h	Cumulative drug delivered $\propto$ current passed
Tamada & Comyns 2005	Anodal and cathodal extraction of glucose. Human *in vivo*, AgCl electrodes	Off period is useful for sample processing	15 mins on, 5 mins off, for 5 h	No control comparison given

This limited set of studies broadly shows that cumulative drug delivered is a function of total current passed, as expected in accordance with Faraday's law. That said, the precise effect of pulsatile iontophoresis is likely to depend on the permeant involved, and on the current profile (Hirvonen *et al*. 1995).

The current profile can also be modified by intermittently reversing the polarity of the electrodes. This strategy was proposed by Lattin and Spevak (1983) as a way to extend the lifetime of an iontophoretic delivery device. They observed that an electrode has a finite lifetime, due to electrochemical factors, or due to drug depletion. Therefore, the lifetime of a delivery device could be doubled simply by placing drug at both electrodes, and running each electrode in turn as the delivery electrode, either each until exhaustion, or in 5 minute cycles, for example. The previously mentioned possibility of reduction of skin irritation due to depolarisation of the skin would also apply. Despite these possible advantages, the effect of reversing polarity (RP) is not well explored (table 5.3).

Table 5.3: Some previous studies using RP iontophoresis, meaning that the polarity of the electrodes was reversed intermittently. Studies where the reversal was on a Hertz scale are not described.

Authors and year	Details	Rationale	Timings	Outcome
Delgado-Charro & Guy 1995	Delivery of nafarelin. Hairless mouse, AgCl electrodes	Release of drug reservoir from skin	Every 6h for 24h	RP flux not different from pulsatile current
Santi & Guy 1996b	Extraction of mannitol. Hairless mouse, AgCl electrodes	1. Off period is used for sample processing 2. Reduce iontophoresis-induced inflammation	Every 15 mins for 2 h	Flux not different from control
Tamada & Comyns 2005	Extraction of glucose, human *in vivo*	Simultaneous extraction from 2 skin sites increased statistical power of experiment.	Every 2.5 or 7.5 mins for 5 h	RP gave better correlation with blood glucose than continuous current

The possible benefits of placing drug at both the anode and the cathode, and delivering from each simultaneously, is hinted at in work by Lopez *et al*. (2003). The flux of zwitterionic aminolevulinic acid delivered from both electrodes simultaneously during direct, constant current iontophoresis was greater than the flux from either electrode alone.

Both pulsed iontophoresis and RP might be expected to result in some mobilisation of the BUP reservoir in the skin (Delgado-Charro & Guy 1995). RP was investigated in this thesis because in contrast to pulsatile delivery, during RP iontophoretic delivery occurs continuously.

As a cation, BUP is delivered by ER from the anode. Delivery of BUP from the cathode is therefore termed 'wrong-way iontophoresis' (Pikal 2001). It was hypothesised that during the (wrong-way) cathodal period of RP, BUP could be released from the skin either passively or aided by EO (Delgado-Charro & Guy 1995). It has been shown previously that if an accumulation of lipophilic cations is large enough, it can not only cancel the negative charges of the skin, but actually confer a net positive charge to it; consequently, EO in the cathode-to-anode direction becomes dominant (Delgado-Charro & Guy 1994). Though it was shown in sections B and C of chapter 4 that BUP reduced EO in the anode-to-cathode direction, it was not known to what extent EO in the cathode-to-anode direction was occurring. EO in the cathode-to-anode direction was not measured in those experiments because of the analytical difficulty of analysing very low concentrations of ACM in the donor solution in the presence of 1 mg/ml BUP.

Figure 5.1 illustrates the theoretical mechanisms influencing BUP flux under a regimen of RP. Up to now, experiments in this thesis have been carried out using a 2-compartment cell with the anode in the donor compartment and the cathode in the receiver compartment. In this section it was necessary to use a 3-compartment cell. A central compartment containing the receiver solution was sandwiched between two separate electrode compartments (figure 5.2).

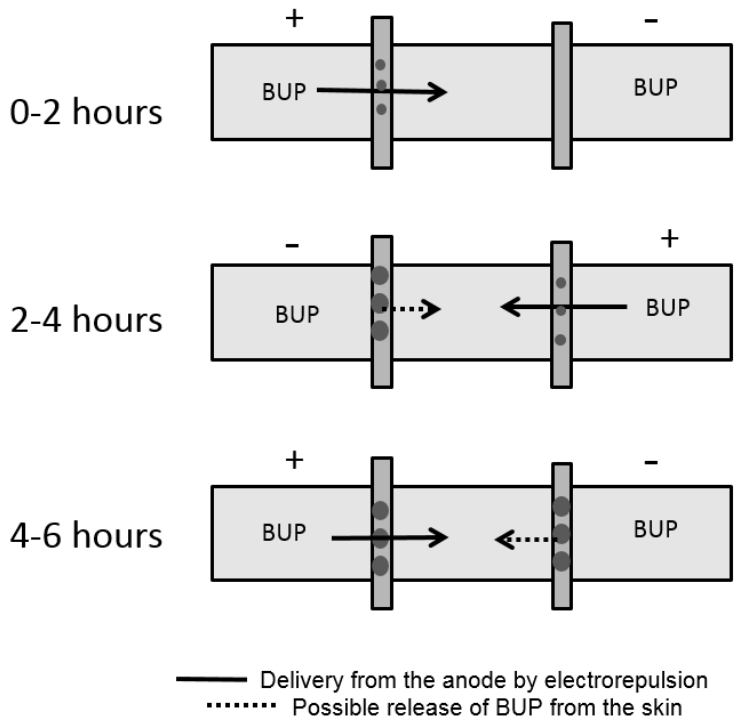

Figure 5.1: Illustration of the movement of BUP during iontophoresis when the polarity of the electrodes is reversed every 2 hours. Experiments were performed in a 3-compartment cell, with the receiver solution in the central compartment. During 0-2 hours, BUP is delivered from the anode and begins to accumulate in the skin on the left. During 2-4 hours, BUP is delivered from the anode and begins to accumulate in the skin on the right. Simultaneously, BUP in the skin on the left can move into the central subdermal compartment due to passive diffusion and the influence of cathode-to-anode EO. During 4-6 hours, BUP is delivered from the anode. Simultaneously, BUP in the skin on the right can move into the central subdermal compartment due to passive diffusion and the influence of cathode-to-anode EO.

Effect of molar fraction

In the last section of this chapter, the effect of molar fraction of drug in the donor on flux is revisited. In section A of chapter 4, it was shown that changing the concentration of NTX in the donor concentration over the range 0.55 to 55 mg/ml while keeping the concentration of BUP fixed had a predictable effect; flux of NTX was proportional to NTX molar fraction. This is explained by co-ion competition and is consistent with studies of co-delivery of sodium and lidocaine (Mudry *et al.* 2006b) and calcium and hydromorphone (Phipps & Gyory 1992). Subsequently, in section C of chapter 4, it was shown that BUP behaved anomalously, and BUP flux did not increase despite a 5-fold increase in BUP concentration in the donor. The molar fraction study in this chapter varies from the previous chapter in two ways. Firstly, having established that NTX is very efficiently delivered by iontophoresis and that far lower donor concentrations than originally tested are required, a concentration range of 0.07 to 0.14 mg/ml NTX was used. Secondly, the previous data for NTX and BUP were collected under different experimental conditions (different current densities and different receptor solutions); the two drugs are now examined under the same conditions.

In this chapter, the combined iontophoretic delivery of NTX and BUP in a situation closer to physiological conditions was assessed, to improve prediction of their behaviour *in vivo*. In order to increase the efficiency of delivery of BUP, and to characterise and try to minimise the effect of the BUP reservoir, the following experimental conditions were tested:

- Section A: Effect of receiver solution
- Section B: Post-iontophoretic release of drug from the skin
- Section C: Effect of reversing polarity (RP) on iontophoresis of NTX and BUP
- Section D: Effect of drug concentration in the donor on iontophoresis of NTX and BUP

2. Methods and materials

The materials and general description of iontophoresis are identical to the previous chapter; refer to page 79.

Section A: Effect of receiver solution

The side-bi-side cells used in this section allowed 0.95 cm^2 of skin to be available for drug transport. The volume of both the donor and receiver solutions was 3.5 ml. The donor solution was 1 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl. pH was adjusted to 5 with NaOH or HCl. The first receiver solution tested was phosphate buffered saline pH 7.4 (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per litre of water). Subsequently, three other receiver solutions were also tested (see table 5.5). The anode was in the donor compartment and the cathode was in the receiver compartment.

A direct, constant current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3 times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. The donor was completely refreshed at 1, 2 and 4 hours. The complete refreshing of the donor and receiver solutions was performed to minimise any pH drift that might occur due to components of the skin leaching out during the experiment. The table below describes the experiments presented in this section.

Table 5.4. Summary of the experimental conditions used to demonstrate the effect of receiver solution on iontophoresis of NTX and BUP.

Donor	Current intensity (current density)	At end of 6h
0.14 mg/ml NTX 1.0 mg/ml BUP & 0.5 mg/ml ACM in 60 mM Tris pH 5.0	0.285 mA (0.3 mA/cm^2)	Tape stripping for receiver solutions A and B only (defined in table 5.5)

Table 5.5. Composition of the receiver solutions used in this section. (Receiver solution A was used in section B of chapter 4 and is shown for comparison.)

Receiver solution	Tris HCl (mM)	Tris base (mM)	Phosphate buffer (mM)	Sodium chloride (mM)	Total molarity (mM)
A Tris pH 5.0	60	-	-	-	60
B Phosphate buffered saline pH 7.4	-	-	15	138	153
C Tris pH 7.4	16	126	-	-	142
D Phosphate buffer pH 7.4	-	-	15	-	15
E NaCl pH 6.0	-	-	-	138	138

Section B: Post-iontophoretic release of drug from the skin

The side-bi-side cells used in this section allowed 0.95 cm^2 of skin to be available for drug transport. The volume of both the donor and receiver solutions was 3.5 ml. The donor solution was 1 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl. pH was adjusted to 5 with NaOH or HCl. The receiver solution for the first 6 hours was PBS pH 7.4.

A direct, constant current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3

times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. The donor was completely refreshed at 1, 2 and 4 hours. At 6 hours, when the current was switched off, all drug was removed from the donor chamber by rinsing, and replaced with PBS pH 7.4, and the receiver solution was exchanged for 60 mM TrisHCl buffer pH 4.0 (see discussion). The receiver was then sampled at 7, 8, 9, 23 and 24 hours. It follows that any drug detected in the receiver at these times must have originated in the skin itself. The table below describes the experiment presented in this section.

Table 5.6: Summary of the experiment to observe post-iontophoretic passive release of NTX, BUP and ACM.

Donor (for first 6 hours)	Receiver (for first 6 hours)	Receiver (from 6 to 24 hours)	Current intensity (current density)	At end of 6h
0.14 mg/ml NTX 1.0 mg/ml BUP & 0.5 mg/ml ACM in 60 mM Tris pH 5.0	PBS pH 7.4	Tris pH 4.0	0.285 mA (0.3 mA/cm^2)	Passive release of NTX, BUP & ACM

Section C: Effect of reversing current polarity

In this section, a 3-compartment cell (PermeGear, US) was used (see figure 5.2). A central compartment held the receiver solution, whilst there were 2 outer electrode chambers. As previously, the cell was held together in a clamp; 0.95 cm^2 of each piece of skin was exposed. The volume of each of the three chambers was 3.5 ml. The donor solution was 0.5 mg/ml BUP, 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl pH 5.0 (pH was adjusted with NaOH or HCl). The receiver solution was PBS pH 7.4.

Figure 5.2: Photograph of a 3-compartment cell. The central compartment is the receiver compartment, the two outer compartments are the electrode compartments.

In one set of experiments, the polarity of the electrodes was reversed every 2 hours. For example, the electrode that ran as an anode for 0-2 hours ran as a cathode for 2-4 hours. The choice of a 2 hour period between polarity reversals was arbitrary. The duration of the experiment was extended to 8 hours (from 6 hours in previous sections) to enable equal sessions of each electrode acting as an anode and as a cathode. The electrode compartment which started as the anode was denoted as '+/-', and the electrode which started as the cathode was denoted as '-/+'. Donor solution was placed in both electrode compartments.

As a control, a second experiment was run where the polarity was not changed during the experiment ('standard' iontophoresis). In these cells, donor solution was only placed in the anodal compartment, whilst the cathode compartment contained Tris 60 mM pH 5.0. No difference in flux was expected by using a 3-compartment set-up rather than a 2-compartment set-up (Harper Bellantone *et al*. 1986). This control was performed to measure fluxes over 8 hours, to match the duration of the RP experiment; previous experiments were 6 hours in duration.

A direct, constant current of 0.285 mA was passed for 8 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3 times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. Both the donor solutions were completely refreshed at 1, 2 and 4 hours. In this section, tapes were grouped together as follows for extraction: 1, 2, 3-5, 6-8 and 9-12. The table below describes the experiments presented in this section.

Table 5.7. Summary of the experiments on the effect of intermittent reversing of the polarity of the electrodes.

	Standard iontophoresis	Reversing polarity
Current intensity (current density)	0.285 mA (0.3 mA/cm^2)	
Electrode chamber A	0.14 mg/ml NTX, 0.5 mg/ml BUP & 0.5 mg/ml ACM in 60 mM Tris pH 5.0	
Central subdermal compartment	PBS pH 7.4	
Electrode chamber B	60 mM Tris pH 5.0	0.14 mg/ml NTX 0.5 mg/ml BUP in 60 mM Tris pH 5.0
At end of 8h	Tape-stripping then tissue extraction	

Section D: Effect of concentration of drug in the donor solution

Excised dorsal pig skin was stretched across the aperture of a 2-compartment side-bi-side glass cell (PermeGear, US) held together in a clamp; 0.95 cm^2 of skin was exposed. The outer surface of the skin was bathed in donor solution (3.5 ml). The underside of the skin was bathed in receiver solution (3.5 ml). Both compartments were stirred by magnetic bars. The anode was placed in the donor chamber, the cathode was placed in the receiver chamber. The donor solution was 0.5 or 1 mg/ml BUP, 0.07 or 0.1415 mg/ml NTX and 0.5 mg/ml ACM prepared in 60 mM TrisHCl. pH was adjusted to 5 with NaOH or HCl. The receiver solution was PBS pH 7.4.

A direct, constant current of 0.285 mA was passed for 6 hours, during which the receiver was sampled every hour for analysis by HPLC. The receiver was completely refreshed 3 times (at 1, 2, and 3 hours), at the remaining samples, 1 ml was sampled from the receiver and replenished. The donor was completely refreshed at 1, 2 and 4 hours. The table below describes the experiments presented in this section.

Table 5.8. Summary of the experiments on the effect of concentration of NTX and BUP in the donor solution.

Donor			Receiver	Current intensity (current density)	At end of 6h
NTX (mg/ml)	BUP (mg/ml)	Background electrolyte			
0.07	0.5	0.5 mg/ml ACM in 60 mM Tris pH 5.0	PBS pH 7.4	0.285 mA (0.3 mA/cm^2)	Tape-stripping then tissue extraction
0.14	0.5				
0.14	1.0				

Analytical and statistical methods are identical to the previous chapter; refer to pages 80-82.

3. Results and Discussion

In chapter 4, the experiments in sections B, C and D were performed using a so-called symmetrical set-up, with identical background electrolyte solutions used in the donor and receiver compartments of each cell. This set-up is common, particularly when examining the effect of pH, as it can facilitate interpretation of the results (Sieg *et al*. 2004). However, an influence of receiver solution on the system, specifically an effect of the pH, may be expected. For that reason, in this chapter it was preferred to use a receiver solution that would closer mimic the *in vivo* situation; the effect of this was assessed. Thereafter, the strategy of intermittent reversal of the polarity of the electrodes was examined. Lastly, the effect of independently varying the concentration of NTX and BUP in the donor solution is reported.

Section A: Effect of receiver solution

The importance of an appropriately selected receiver solution in passive skin experiments has been described (Bronaugh & Stewart 1984; Scott & Ramsey 1987; Kou *et al.* 1993). The effect of receiver solution, and particularly the solubility of the permeant in the receiver solution, on iontophoretic flux, where passive diffusion and partitioning are not the major driving forces, is less often discussed (Phipps & Gyory 1992). The challenge resides in establishing the key parameters that determine the iontophoretic flux *in vitro*, to achieve better prediction of *in vivo* fluxes. In the case of lipophilic compounds such as BUP, an added difficulty *in vitro* lies in finding a receiver solution in which the permeant has adequate solubility.

In the case of BUP, previous researchers have taken different approaches to this problem. Robson (1988) and Wang *et al.* (2009) (both passive) used receiver solutions containing 50% ethanol. This obviously increases the solubility of BUP in the receiver, but may overestimate drug flux, as ethanol permeates into the skin during the experiment, and reduces the skin's barrier function. For example, Kou *et al.* (1993) showed a 10-fold increase in the passive flux of nicardipine following inclusion of 50 % ethanol in the receiver. Similarly Hirvonen *et al.* (1996) showed a doubling of the passive flux of mannitol following the inclusion of 90% ethanol in the receiver solution. Roy *et al.* (1994, passive), Stinchcomb *et al.* (1996, passive), Bose *et al.* (2001, iontophoresis), and Fang *et al.* (2002, iontophoresis) addressed the issue by using receiver solutions with pH 6 or 6.4 as the solubility of BUP increases with decreasing pH. The concern is that those pHs were chosen arbitrarily; the interstitial fluid of the subdermal environment is close to pH 7.4.

The first receiver solution tested in this chapter was PBS pH 7.4. It is commonly used as a receiver solution in both passive and iontophoretic skin permeation experiments. It has a pH relevant to the physiological situation, it is not expected to alter the barrier function of the skin, and lastly, it has physiological levels of chloride. As chloride is the main endogenous anion competing for charge-carrying when delivering a positively charged drug, use of a receiver solution that does not contain chloride would be likely to greatly overestimate cationic drug flux (Burnette & Ongpipattanakul 1987; Mudry *et al*. 2006a). The objective of this experiment was to determine the effect, if any, of switching to a receiver solution of PBS pH 7.4. Table 5.9 and figures 5.3 and 5.4 show the results.

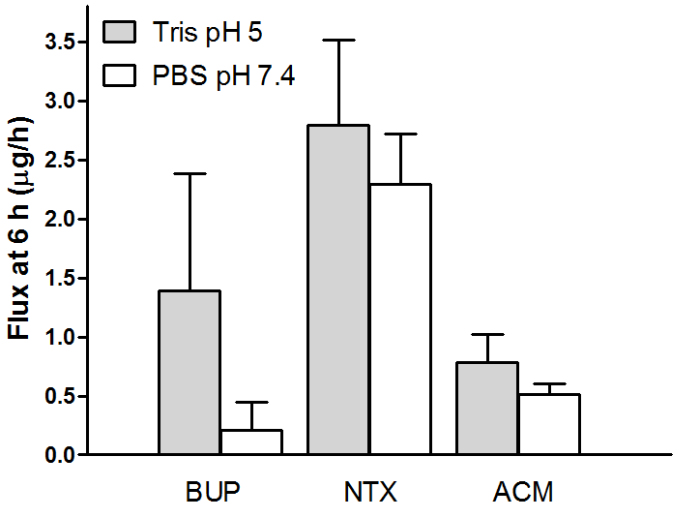

Figure 5.3: Iontophoretic fluxes (mean + standard deviation) of BUP, NTX and ACM. Receiver solution was either Tris pH 5 or PBS pH 7.4 (n = 6 and 6). All other parameters were identical. The donor solution was 0.14 mg/ml NTX, 1.0 mg/ml BUP and 0.5 mg/ml ACM, in 60mM Tris pH 5.0. The current intensity was 0.285 mA.

A clear effect of receiver solution on BUP flux was observed (figure 5.3). The flux of BUP into a PBS receiver solution was significantly lower ($p < 0.05$) than into Tris buffer. This result was not expected. It had been anticipated that if anything, the flux of BUP might be *higher* with PBS pH 7.4 compared to Tris pH 5 because of an expected increase in the contribution from EO at the higher pH (Kim *et al.* 1993).

The decrease in BUP flux associated with the use of a PBS receiver solution could not be explained by absence of sink conditions. Sink conditions are defined as ≥ 10-fold difference between observed concentration of the drug in the receiver solution, and the maximum aqueous solubility of the drug in the receiver solution. The solubility of BUP in PBS pH 7.4 is 45 µg/ml (Robson 1988), while sample concentrations were all < 1 µg/ml.

Additionally, in contrast to sections B and C of chapter 4 the flux of BUP was not reduced due a lower EO flow as ACM fluxes were not significantly different for the two receiver solutions used. Furthermore, neither the amount of BUP recovered, nor the concentration of BUP in the stratum corneum (table 5.9) were significantly different for the two receiver solutions.

Table 5.9: Amount of BUP recovered from the stratum corneum. Receiver solution was either Tris pH 5 or PBS pH 7.4. No NTX was quantifiable beyond tape 1. Values are mean ± standard deviation.

Receiver solution	BUP recovered from tapes (µg)	Stratum corneum removed (µg)	Concentration of BUP in stratum corneum (µg/mg)
Tris pH 5.0	23.3 ± 5.1	408 ± 214	81.2 ± 62.9
PBS pH 7.4	24.1 ± 5.5	587 ± 80	42.2 ± 12.1

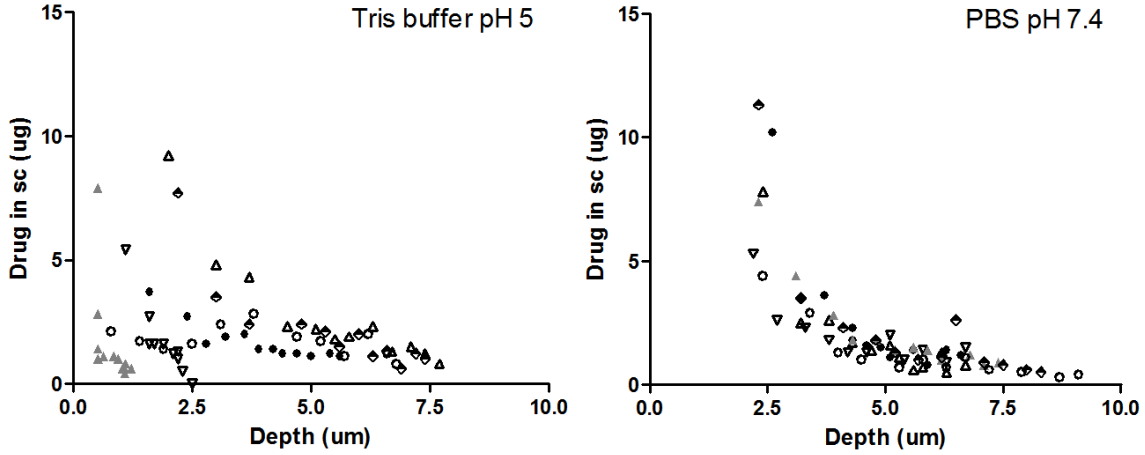

Figure 5.4: Amount of BUP in the stratum corneum (mean + standard deviation) as a function of depth. The donor solution was 0.14 mg/ml NTX, 1.0 mg/ml BUP and 0.5 mg/ml ACM, in 60mM Tris pH 5.0. The current intensity was 0.285 mA. **Left**: receiver solution was Tris buffer pH 5 (n = 6). **Right**: receiver solution was PBS pH 7.4 (n = 6).

Therefore, overall, no immediate explanation for the observed difference in BUP fluxes for the two receiver solutions was apparent. The two receiver solutions compared here differ in buffer type, ionic strength, and pH, all of which can have an influence on iontophoretic flux (Riviere & Heit 1997; Tamada & Comyns 2005). Therefore, three more receiver solutions (C, D and E, defined in table 5.10) were tested in an attempt to understand the observed difference. Figure 5.5 and table 5.10 show the results.

The first hypothesis to explain the reduction in BUP flux observed upon adopting a receiver solution of PBS was that it was an effect of buffer type. To test this idea, a receiver solution with the same pH and very similar ionic strength to PBS (receiver solution B), but with a Tris buffer system, was prepared (receiver solution C). However, there was no indication that buffer type was important; BUP flux using receiver solution C was close to when using PBS (receiver solution B).

Table 5.10: Fluxes of NTX, BUP and ACM into different receiver solutions. All other parameters were identical. Donor solution was 0.14 mg/ml NTX, 1.0 mg/ml BUP and 0.5 mg/ml ACM, in 60mM Tris pH 5.0. The current intensity was 0.285 mA.

			NTX		BUP		ACM	
	Receiver solution	n	6h cumulative delivery (µg)	Flux at 6h (µg/h)	6h cumulative delivery (µg)	Flux at 6h (µg/h)	6h cumulative delivery (µg)	Flux at 6h (µg/h)
A	Tris pH 5.0	6	8.2 ± 3.1	2.8 ± 0.7	2.2 ± 2.1	1.4 ± 1.0	2.5 ± 0.9	0.8 ± 0.2
B	PBS pH 7.4	6	7.0 ± 1.3	2.3 ± 0.4	0.9 ± 1.1	0.2 ± 0.2	1.7 ± 0.2	0.5 ± 0.1
C	Tris pH 7.4	2	5.0, 10.8	1.7, 3.2	0.0, 0.3	0.0, 0.3	1.5, 3.1	0.4, 0.7
D	Phosphate buffer pH 7.4	5	16.5 ± 7.7	5.2 ± 1.9	0.1 ± 0.2	0.1 ± 0.2	4.5 ± 2.1	1.5 ± 0.6
E	NaCl pH 6	4	8.4 ± 0.03	2.3 ± 0.1	4.9 ± 4.9	2.0 ± 1.8	3.1 ± 0.8	0.8 ± 0.2

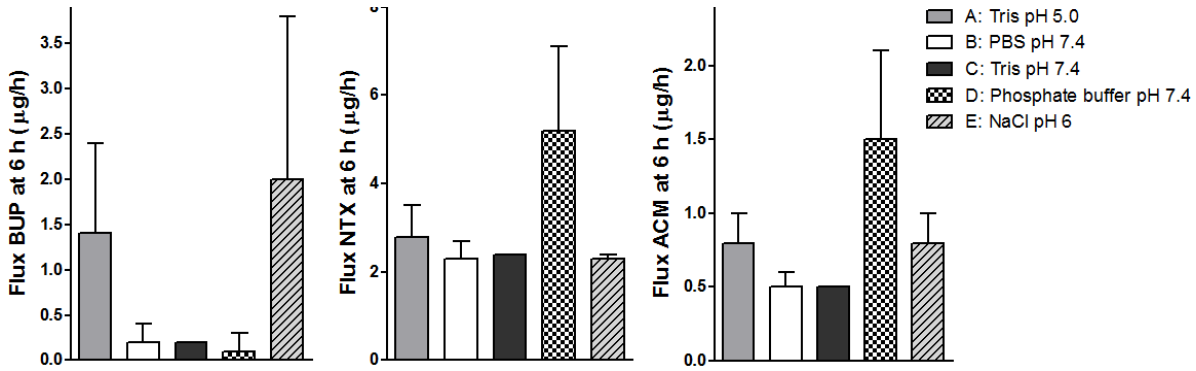

Figure 5.5: Fluxes of BUP, NTX and ACM (mean + standard deviation) into different receiver solutions. The donor solution was 0.14 mg/ml NTX, 1.0 mg/ml BUP and 0.5 mg/ml ACM, in 60mM Tris pH 5.0 (n = 6, 6, 2, 5, and 4).

The second hypothesis to explain the reduction in BUP flux observed upon adopting a receiver solution of PBS was that it was an effect of increased counter-ion competition. The previously used Tris buffer had only 60 mM chloride, whereas PBS contains 138 mM chloride. Chloride in the receiver solution is a counter-ion that competes with BUP (and NTX) to carry charge. To test this hypothesis, receiver solution D (phosphate buffer pH 7.4 but

with no sodium chloride) was prepared. Again, the flux of BUP using receiver D was very similar to that observed when using PBS; the different level of chloride did not seem to effect the behaviour of BUP. In contrast, the flux of NTX was higher using receiver solution D than any other tested. This could be explained by the following 2 factors:

1.The absence of chloride ions in receiver solution D. In the absence of chloride, phosphate is the major anion in the receiver fluid, but as it is larger and less mobile than chloride, it is less good at competing with NTX for the available charge (Phipps & Gyory 1992). It was not the aim of this section to investigate competition with counter-ions, but this data does highlight how the efficiency of iontophoretic delivery of cations will always be limited by the presence of ubiquitous endogenous chloride in the subdermal compartment *in vivo* (Mudry *et al*. 2006a). Furthermore, it is expected that less mobile ions (such as BUP), respond proportionally less than more mobile ions (such as NTX) to changes in the composition of counter-ions in the receiver solution (Mudry 2006c).

2. The low ionic strength (15 mM) of receiver solution D. An inverse relationship between ionic strength and EO has been documented (Tamada & Comyns 2005). The corresponding increase in the flux of ACM is consistent with the increased flux of NTX being driven by EO.

Lastly, receiver solution E (sodium chloride only) was prepared; this is commonly used as a receiver solution. Unexpectedly, the flux of BUP returned to the levels previously observed with Tris buffer, and was 10 times higher than with PBS pH 7.4. Receiver solution E was unbuffered and had a pH of 6. Higher pHs are expected to result in a greater contribution to flux from EO via an effect on skin charge (Marro *et al.* 2001b). However, as well as effecting the skin, the pH also effects the drug, and the aqueous solubility of BUP drops dramatically between pH 5.0 and pH 7.4 (from 12 mg/ml to 0.045 mg/ml, Robson 1988). It was hypothesised therefore, that the low fluxes observed into PBS pH 7.4 were due to BUP favouring the lipid environment of the skin over the aqueous receiver solution, and therefore only partitioning into the receiver solution very slowly. If this is the case, a significant amount of BUP should accumulate in the skin that did not partition into the receiver solution. This hypothesis was explored in section B.

Section B: Post-iontophoretic release of drug from the skin

To test whether BUP, which has pH-dependent aqueous solubility, can move freely into a Tris buffer of pH 5, but not into PBS pH 7.4, an experiment was carried out in which post-iontophoresis fluxes were measured. ‘Post-iontophoretic flux’ is simply the flux of drug that

occurs after the current is terminated (Thysman *et al.* 1994; Santi & Guy 1996a; Bose *et al*. 2001).

Post-iontophoretic flux has been measured to monitor passive desorption of a drug from the skin. This can be achieved by termination of the current, removal of drug from the donor compartment, and continued sampling of the receiver solution (Delgado-Charro & Guy 1995). Post-iontophoretic flux has also been used to measure an iontophoresis-induced increase in passive permeability of the skin. This can be achieved by monitoring the post-iontophoresis fluxes of water (Kim *et al*. 1993), or mannitol (Kim *et al*. 1993; Santi & Guy 1996b) for example.

Post-iontophoretic flux was measured in this section to see the effect of pH of the receiver solution on passive release of NTX and BUP from the skin into the receiver chamber. A standard 6 hour experiment was performed, but at 6 hours, current was terminated, donor solution was removed from the donor compartment, and replaced with buffer only (PBS pH 7.4). The receiver solution was replaced with Tris buffer 60 mM pH 4.0. This was chosen because it was anticipated that BUP would be able to move freely into this receiver solution. At pH 4.0, the aqueous solubility of BUP is ~19 mg/ml (Robson 1988). Table 5.11 and figure 5.6 show the results.

Table 5.11: Amount of NTX, BUP and ACM permeated during iontophoresis, and subsequently released passively from the skin itself (mean ± standard deviation). The donor solution was 0.14 mg/ml NTX, 1.0 mg/ml BUP and 0.5 mg/ml ACM, in 60mM Tris pH 5.0. The current intensity was 0.285 mA. The receiver solution between 0-6 hours was PBS pH 7.4 and the receiver solution between 6-24 hours was Tris pH 4.0.

		NTX		BUP		ACM	
Receiver solution	n	Cumulative delivery (µg)	Flux at 6 or 24 h (µg/h)	Cumulative delivery (µg)	Flux at 6 or 24 h (µg/h)	Cumulative delivery (µg)	Flux at 6 or 24 h (µg/h)
Iontophoresis 0 to 6 hours	6	8.4 ± 1.6	2.6 ± 0.5	0.7 ± 0.4	0.4 ± 0.2	1.8 ± 0.4	0.5 ± 0.1
Post-iontophoresis 6 to 24 hours	6	13.0 ± 0.8	0.1 ± 0.1	23.9 ± 6.3	1.6 ± 0.3	3.2 ± 0.4	0.1 ± 0.0

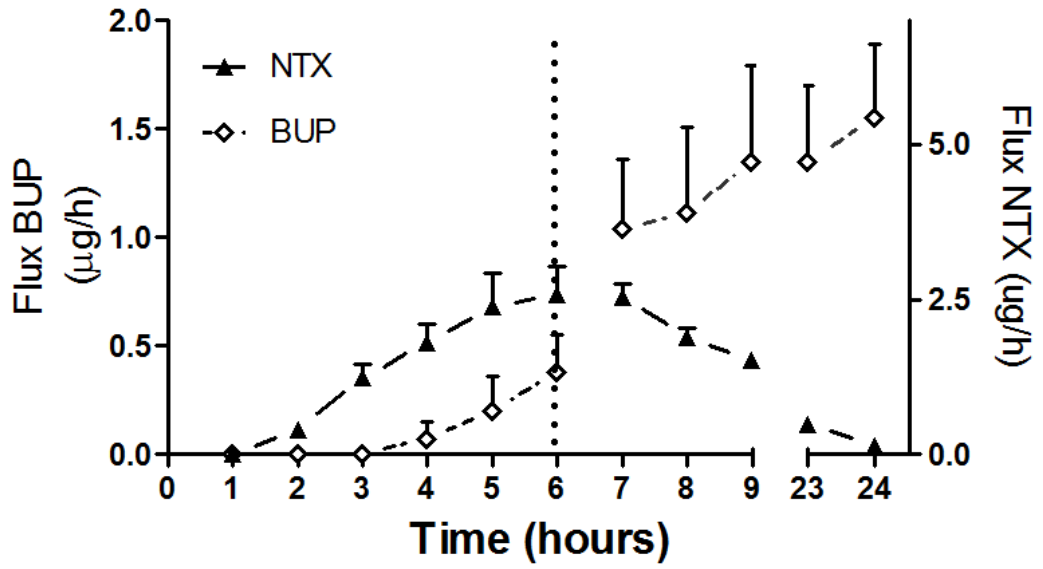

Figure 5.6: Iontophoretic fluxes (0-6 hours), and post-iontophoretic fluxes (6-24 hours) of BUP and NTX (mean + standard deviation, n = 6). The dotted line indicates termination of current, removal of donor solution, and switching to a receiver solution (pH 4.0) in which BUP is relatively soluble.

As expected, from 6 hours onwards the release of NTX from the skin slowed, presumably as the amount of NTX in the skin was depleted. In striking contrast, the rate of release of BUP from the skin into the receiver solution actually *increased* when the current was switched off. To reiterate, there was no drug in the donor compartment after 6 hours, so fluxes represent drug being released from the skin. These data confirm that a substantial amount of BUP deposits in the skin during iontophoresis, and that BUP distributed into the receiver solution far more easily when Tris pH 4.0 was used than when PBS pH 7.4 was used. This provides a credible explanation for the low flux of BUP with a receiver solution of pH 7.4 compared to a receiver solution of pH 5.0 observed in section A.

It is possible that slow partitioning of BUP into a receiver solution of PBS pH 7.4 might result in underestimation of *in vivo* fluxes. To minimize underestimation of BUP fluxes, the drug in the viable tissue at the end of the experiment was determined from this point.

Ideally, the suitability of any receiver solution used *in vitro* would be confirmed by demonstrating correlation with *in vivo* drug fluxes. Such a 'back-to-front' approach was taken by Gyory *et al.* (1997) when investigating the iontophoretic delivery of fentanyl. It was reported that an excellent *in vivo: in vitro* correlation was achieved by selecting the appropriate receiver solution, and that the ionic strength of the receiver solution was of particular importance (no values given).

Section C: Reversing polarity

During this experiment BUP and NTX were present in both donor compartments, and the polarity was reversed every 2 hours. Therefore, BUP and NTX were delivered by anodal iontophoresis (as until now) and simultaneously by cathodal (wrong-way) iontophoresis. It was hypothesised that the delivery of BUP could take place not only from the donor solution but additionally from any skin reservoir built up in the previous two hour period. The objective of these experiments was to determine the influence of RP on the flux of NTX and BUP, on EO, and on the amount of drug in the skin. Tables 5.12, 5.13 and 5.14, and figures 5.7 and 5.8 show the results.

Table 5.12: Amount of NTX recovered (mean ± standard deviation) from the viable skin following 8 hours of either standard iontophoresis, or after reversing the polarity of the electrodes every 2 hours over an 8 hour period. No NTX was quantifiable beyond tape 1. Letters in superscript indicate pairs of values are significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

	NTX in viable skin (µg)		Total NTX in skin (µg)
	+/-	-/+	
Standard iontophoresis	8.2 ± 0.7[a,b]	n/a	8.2 ± 0.7
Reversing polarity	2.3 ± 1.1[a,c]	4.8 ± 0.4[b,c]	7.1 ± 0.7

Table 5.13: Amount of BUP (mean ± standard deviation) recovered from the skin following iontophoresis with standard iontophoresis for 8 hours, or after reversing the polarity of the electrodes every 2 hours over an 8 hour period. Letters in superscript indicate pairs of values are significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

	BUP in viable skin (µg)		BUP in tapes (µg)		Stratum corneum removed (µg)	
	+/-	-/+	+/-	-/+		Total BUP in skin (µg)
Standard iontophoresis	39.6 ± 9.2[a,b]	n/a	24.5 ± 5.6[d]	n/a	758 ± 150	64.0 ± 5.6
Reversing polarity	11.9 ± 2.9[a,c]	18.0 ± 1.0[b,c]	10.5 ± 2.1[d,e]	19.6 ± 1.8[e]	650 ± 127	60.0 ± 7.6

It was hoped that employing RP would reduce the depot of BUP in the skin. Indeed, under the RP regimen BUP was found at lower amounts ($p < 0.05$) in the viable skin, and in the stratum corneum of the +/- skin site (the chamber that acted first as the anode). However, bearing in mind that when employing RP two skin sites instead of one contained drug, there was no difference in total BUP in the skin compared to standard iontophoresis. Interestingly, the skin site that started as the anode (+/-) contained significantly less ($p < 0.05$) BUP and NTX than the skin site that started as the cathode (-/+). It is possible that during 6-8 hours, some drug moved from the +/- skin into the receptor solution by passive diffusion.

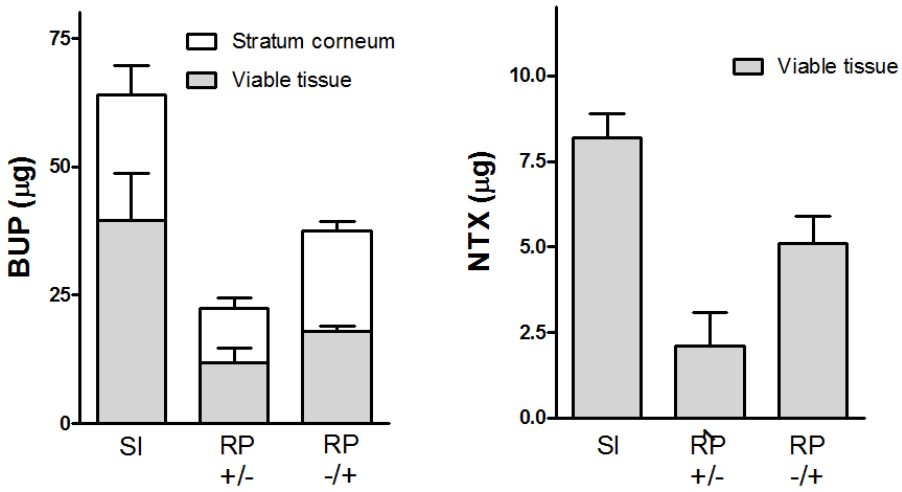

Figure 5.7: Total recovery of BUP and NTX (mean + standard deviation) from the skin following standard iontophoresis (SI, n = 3) or a regimen of reversing polarity (RP, n = 3); +/- denotes the skin compartment initially providing anodal delivery of the drugs; -/+ denotes the skin compartment initially providing cathodal delivery of the drugs.

In this experiment, ACM was only placed in one donor chamber – the chamber that acted first as the anode (the +/- chamber). Therefore the flux of ACM between 0-2 hours was a measure of anode-to-cathode EO, and flux of ACM between 2-4 hours was a measure of cathode-to-anode EO, and so on.

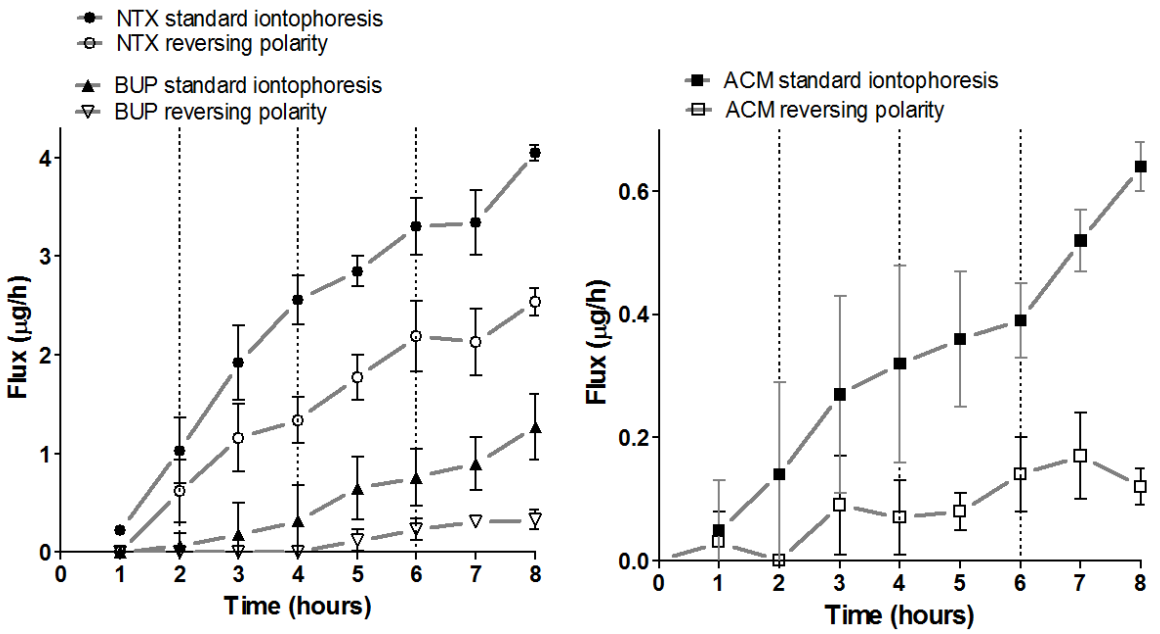

Figure 5.8: Iontophoretic fluxes (mean + standard deviation) of BUP and NTX (**left**) and ACM (**right**) under standard iontophoresis or under a regimen of reversing polarity (n = 3 and 3), as a function of time. The donor solution was 0.14 & 0.5 mg/ml NTX & BUP respectively, and 0.5 mg/ml ACM, in 60mM Tris pH 5.0. The current intensity was 0.285 mA. The vertical dotted lines indicate reversal of the polarity of the electrodes every 2 hours.

It had been anticipated that a 'saw-tooth' pattern might have been generated, as observed by Santi & Guy (1996b) when measuring the extraction of mannitol into the two electrode compartments while reversing the polarity of the electrodes every 15 minutes. No clear pattern of that type was observed here (figure 5.8), implying either that anodal and cathodal EO were similar in magnitude, or that flux of ACM driven by EO did not respond immediately when the polarity was changed. Indeed, Kim *et al*. (1993) reported that passive fluxes of mannitol and water, driven by iontophoresis-induced permeability of the skin, were observed for several hours after termination of current.

The flux of ACM was lower during the RP regimen than during standard iontophoresis. One explanation for this is that reversing the polarity every 2 hours was too frequent and did not allow time to EO to 'get going'. However, this explanation is not consistent with the responsiveness of mannitol flux to polarity reversal observed in the Santi & Guy work (1996b), nor with the observation that the GlucoWatch®, a device for diabetic patients that utilised EO to extract glucose across the skin (Sieg *et al*. 2005), reversed polarity every 20 minutes, and was able to extract glucose (by EO alone) in line with blood concentrations. However, those experiments were not influenced by confounding effects on EO as observed here with BUP. Furthermore, mannitol and glucose are both more hydrophilic than ACM (ACM logP 0.46, 151 Da), and therefore less likely to partition in the skin. If ACM

partitions in the skin, flux of ACM may underestimate EO, or be subject to a time lag. In section B of this chapter it was observed that 3.2 µg of ACM accumulated in the skin in 6 hours; compared to 1.8 µg of ACM carried across the skin into the receiver solution over the same period. This is a possible limitation of ACM as a marker of EO, and may contribute to the lack of saw-tooth effect observed here. In any case, there is no evidence that cathode-to-anode EO was occurring, or that BUP was delivered by cathode-to-anode EO.

Disappointingly, the fluxes of BUP and NTX at 8 hours were significantly lower under the RP regimen than under standard iontophoresis. It had been postulated that EO in the cathode-to-anode direction might contribute to total flux; no evidence of such an effect was observed. This finding is not inconsistent with other studies (Hirvonen *et al.* 1995; Delgado-Charro & Guy 1995) who concluded that total transport was determined by the duration of current passed in the conventional anode-to-cathode direction.

Most likely, this was a consequence of flux under the RP regimen being subject to two successive lag times, one for each skin site. This is illustrated in tables 5.15 and 5.16. Using the observed fluxes from the standard iontophoresis experiment, the flux under a regimen of reversing polarity if drug was only delivered from the anode was predicted. Predicted and observed final fluxes were in close agreement. This indicates that the cumulative drug delivered is controlled by the current passed from the anode; no advantage of RP over standard iontophoresis has been demonstrated.

Table 5.14: Iontophoretic fluxes of NTX, BUP and ACM (mean ± standard deviation) with standard iontophoresis or with intermittent reversing of the polarity of the electrodes. Donor was 0.14 mg/ml NTX, 0.5 mg/ml BUP and 0.5 mg/ml ACM in in 60mM Tris pH 5.0. The receiver solution was PBS pH 7.4. Values are mean ± standard deviation. Letters in superscript indicate pairs of values are significantly different (t-test, $p < 0.05$).

		NTX		BUP		ACM	
	n	8 h cumulative delivery (µg)	Flux at 8h (µg/h)	8 h cumulative delivery (µg)	Flux at 8h (µg/h)	8 h cumulative delivery (µg)	Flux at 8h (µg/h)
Standard iontophoresis	3	19.3 ± 1.2[a]	4.0 ± 0.1[b]	4.1 ± 1.9[c]	1.27 ± 0.33[d]	2.7 ± 0.7[e]	0.6 ± 0.0[f]
Reversing polarity	3	11.7 ± 1.6[a]	2.5 ± 0.1[b]	1.0 ± 0.2[c]	0.33 ± 0.10[d]	0.7 ± 0.3[e]	0.1 ± 0.0[f]

Table 5.15: Predicted contributions to total NTX flux from +/- (the electrode that started as the anode) and -/+ (the electrode that started as the cathode). Prediction based on observed data from standard iontophoresis. For site +/- the first 2 hours of iontophoresis is exactly the same as for the control experiment. During 2-4 hours, when the polarity is reversed, if drug is only transported from the anode, no flux will be observed. During 4-6 hours, the polarity is reversed again, and this is akin to 2-4 hours of the control experiment.

	Observed flux in standard iontophoresis (µg/h)	Predicted flux (µg/h)			Observed flux using reversing polarity (µg/h)
		Contribution from +/-	Contribution from -/+	Total flux	
0 to 2 hours	0.62	0.62	0	0.62	0.31 ± 0.16
2 to 4 hours	2.24	0	0.62	0.62	1.25 ± 0.23
4 to 6 hours	3.07	2.24	0	2.24	1.98 ± 0.24
6 to 8 hours	3.69	0	2.24	2.24	2.34 ± 0.17

Table 5.16: Predicted contributions to total BUP flux from +/- (the electrode that started as the anode) and -/+ (the electrode that started as the cathode). Prediction based on observed data from standard iontophoresis. For site +/- the first 2 hours of iontophoresis is exactly the same as for the control experiment. During 2-4 hours, when the polarity is reversed, if drug is only transported from the anode, no flux will be observed. During 4-6 hours, the polarity is reversed again, and this is akin to 2-4 hours of the control experiment.

	Observed flux in standard iontophoresis (µg/h)	Predicted flux (µg/h)			Observed flux in standard iontophoresis (µg/h)
		Contribution from +/-	Contribution from -/+	Total flux	
0 to 2 hours	0.04	0.04	0.00	0.04	0
2 to 4 hours	0.25	0.00	0.04	0.04	0
4 to 6 hours	0.70	0.25	0.00	0.25	0.18 ± 0.07
6 to 8 hours	1.08	0.00	0.25	0.25	0.32 ± 0.05

In the experimental design used here it is not known from which electrode compartment BUP and NTX found in the receiver originate, this could be achieved by the use of radio-labelled drug added to one electrode compartment only.

Section D: Effect of drug concentration in the donor solution

When co-ions are present in the donor it is expected that the flux of a drug will be proportional to the concentration of that drug in the donor solution (Phipps & Gyory 1992; Marro *et al*. 2001b; Mudry *et al.* 2006b). NTX seems to obey this rule, whereas BUP does not.

The aim of this section was to establish the effect of drug concentration in the donor on flux of NTX and BUP, on EO, and on the amount of drug in the skin, in order to enable rational optimisation of the system by selection of an appropriate donor solution. The concentration of BUP was held constant while the concentration of NTX was varied, and then the concentration of NTX was held constant while the concentration of BUP was varied. The fluxes of BUP, NTX and ACM, and drug recovered from the skin, are shown in tables 5.17 and 5.18 and figures 5.9 and 5.10.

Table 5.17: Iontophoretic fluxes of NTX, BUP and ACM (mean ± standard deviation) as a function of donor composition. The background electrolyte in all donor solutions was 60mM Tris pH 5.0. All donor solutions contained 0.5 mg/ml ACM. The current intensity was 0.285 mA. Values are mean ± standard deviation. Letters in superscript indicate pairs of values are significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

mg/ml			BUP		NTX		ACM	
NTX	BUP	n	6 h cumulative delivery (µg)	Flux (µg/h)	6 h cumulative delivery (µg)	Flux (µg/h)	6 h cumulative delivery (µg)	Flux (µg/h)
0.07	0.5	8	0.90 ± 0.78	0.37 ± 0.20	4.87 ± 2.01[b,c]	1.52 ± 0.47[e,f]	2.92 ± 1.40[a]	0.94 ± 0.37
0.14	0.5	7	1.24 ± 0.57	0.61 ± 0.24[a]	11.12 ± 2.86[b,d]	3.37 ± 0.85[e,g]	3.09 ± 1.02[i]	1.01 ± 0.30
0.14	1	9	0.56 ± 0.35	0.31 ± 0.18[a]	7.33 ± 2.02[c,d]	2.18 ± 0.70[f,g]	2.34 ± 1.05[h,i]	0.64 ± 0.25

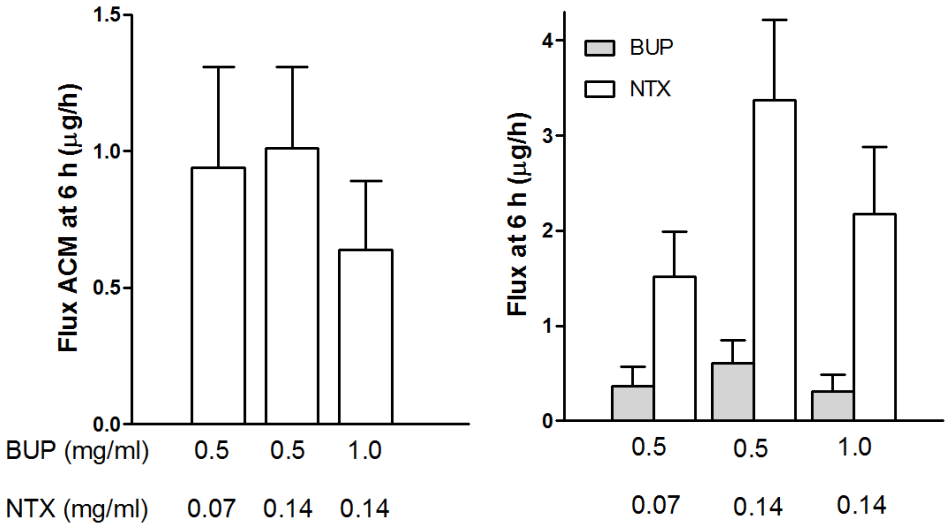

Figure 5.9: Iontophoretic fluxes of NTX, BUP and ACM (mean + standard deviation) as a function of donor composition (n = 8, 7 and 9). The background electrolyte in all donor solutions was 60mM Tris pH 5.0. All donor solutions contained 0.5 mg/ml ACM. The current intensity was 0.285 mA.

Table 5.18 shows the recovery of the two drugs from skin and tapes as the concentration of drug in the donor solution was varied. As seen in section 2 of chapter 4, the amount of BUP found in the skin increased with the concentration of BUP in the donor, while the amount of NTX recovered from the skin was not influenced by donor concentration (of BUP or NTX) over this range.

Table 5.18: Amount of BUP and NTX recovered from the skin (mean ± standard deviation) as a function of donor composition. Letters in superscript indicate pairs of values are significantly different (one-way ANOVA followed by Bonferroni post-tests, $p < 0.05$).

				BUP			NTX
NTX	BUP	n	Stratum corneum removed (µg)	Drug in stratum corneum (µg)	Concentration of drug in stratum corneum (µg/mg)	Drug in viable epidermis (µg)	Drug in viable epidermis (µg)
0.07	0.5	5-8	509 ± 253	17.3 ± 13.3[a]	44.4 ± 31.6	59.0 ± 10.9	10.4 ± 5.1
0.14	0.5	7	528 ± 175	12.8 ± 4.5[b]	27.8 ± 17.0	53.7 ± 28	10.9 ± 4.4
0.14	1	3	564 ± 102	54.4 ± 8.8[a,b]	96.9 ± 7.2	102 ± 6	10.4 ± 1.2

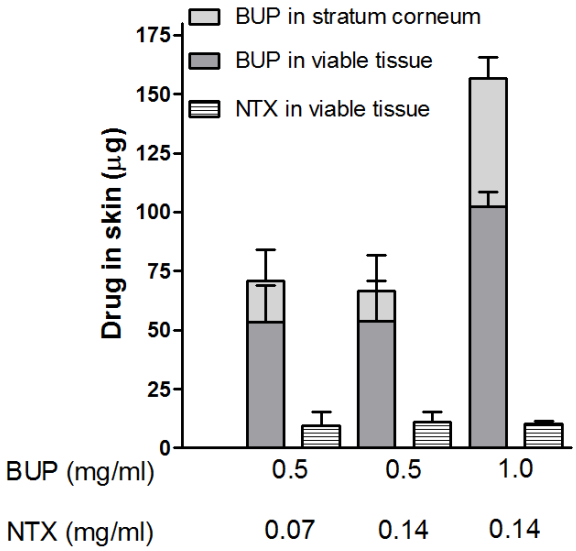

Figure 5.10: Amount of NTX and BUP recovered from the skin (mean + standard deviation) as a function of donor composition (n = 5-8, 7 and 3). The background electrolyte in all donor solutions was 60mM Tris pH 5.0. All donor solutions contained 0.5 mg/ml ACM. The current intensity was 0.285 mA.

As shown in chapter 4, but now confirmed using a more physiologically relevant receiver solution, BUP does not follow the conventional pattern expected of a drug in an iontophoretic system, and a linear relationship between the concentration of BUP in the donor and the flux of BUP was not observed. In fact, when NTX donor concentration was fixed, BUP flux was higher ($p < 0.05$) from a donor containing 0.5 mg/ml BUP than from a donor containing 1.0 mg/ml BUP. Likewise, apparent EO and flux of NTX were decreased ($p < 0.05$) by an increase in BUP donor concentration. Conversely, the amount of NTX in the donor did not influence the flux of BUP. When BUP concentration in the donor was fixed, NTX followed the conventional rules of iontophoresis and NTX flux increased with NTX concentration in the donor.

4. Conclusions of the chapter

In order to closer mimic the *in vivo* situation, a receiver solution of PBS pH 7.4 was adopted in this chapter. It was observed that using PBS pH 7.4 instead of Tris HCl pH 5.0 (whilst fixing all other parameters) had a non-significant effect on all outcomes, except on the flux of BUP, which decreased 5-fold. This was attributed to the pH-dependence of BUP aqueous solubility and resulting slow partitioning of BUP from the skin into a receiver solution of pH 7.4.

The large influence of receiver solution on flux observed in section A shows how care must be taken when predicting *in vivo* fluxes from *in vitro* data. Furthermore, quantification of the

amount of drug in the stratum corneum and in the viable tissue at the end of an iontophoresis experiment can increase understanding of drug behaviour. The significant reservoir of only BUP represents a challenge for this application that will need to be solved before assessing this system *in vivo*.

A strategy of reversing the polarity of the electrodes was investigated. This approach was successful at lowering the amount of BUP recovered from each skin site compared to a standard iontophoresis control. However, under the reversing polarity regimen, as drug accumulates in the skin at both electrodes the total amount of drug recovered from the skin was not different from the control; no benefit to a patient would be expected. Reversing the polarity of the electrodes did not result in increased flux of NTX or BUP.

The amount of BUP in the skin increased with the concentration of BUP in the donor. The BUP depot was associated with a decrease in anode-to-cathode EO, presumably by neutralising negative charges on the skin. Confirming the findings of the previous chapter, a significant 'auto-inhibition' of BUP flux was observed, as well as a decreased NTX flux. When BUP donor concentration was fixed, the effect of NTX donor concentration on NTX flux was predictable.

It has been shown previously that two co-ions delivered simultaneously by iontophoresis can each influence the flux of the other, through ion competition effects. This is often shown with small inorganic cations such as lithium, sodium and potassium (Mudry *et al.* 2006a and b). To the author's knowledge, that one cationic drug (BUP) may negatively influence the simultaneous iontophoretic flux of another cationic drug (NTX) via an effect on EO has not been shown before.

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Chapter 6. General Discussion and Conclusions

In this thesis, experiments were undertaken to assess the viability of an iontophoretic transdermal patch delivering BUP and NTX together as a relapse prevention therapy. The work presented falls into two halves, the pharmacological action of the BUP/NTX combination, and its transdermal delivery.

1.0 mg/kg (but not 0.3 mg/kg) NTX blocked the rewarding properties of 0.3 mg/kg BUP. A range of ratios (between 5:1 and 1:10 NTX:BUP, table 2.1) had been previously reported in rodent studies to achieve blocking of BUP's mu agonsim. We found that blocking was achieved with a ratio of 3:1 NTX:BUP. Our data suggest that the previous clinically administered ratio of 10:1 NTX:BUP may have been an overcautious approach.

0.3 mg/kg BUP with 3.0 mg/kg NTX (but not the same dose of NTX alone) was aversive. The issue of aversion is important because the main drawback of a NTX-alone therapy is that patient adherence is low (Kirchmayer *et al*. 2002) and there is a common conception that this is due at least in part to NTX producing a general dysphoria (Miotto *et al*. 2002). However, our results indicate that NTX alone is not aversive. NTX provides good blockade of heroin, therefore understanding the precise nature of the reported patient dislike for NTX could help to improve use of this drug.

If the aversion observed following administration of 0.3 mg/kg BUP with 3.0 mg/kg NTX is not a simple mu antagonist effect, it might be explained by BUP activity at the NOP receptor. There is great interest in the NOP receptor as it is believed to have 'anti-addictive' properties (Marquez *et al.* 2008; Bebawy *et al*. 2010). The hypothesis of Canestrelli *et al.* (2014) is that when BUP is administered in combination with a mu antagonist (naloxone was used in their study), BUP's activity at the NOP receptor becomes apparent. Their hypothesis was supported by the observation that an NOP antagonist eliminated the aversive effect of BUP and naloxone; a similar test could be performed for BUP and NTX. The importance of the NOP receptor for BUP activity is still unclear, as binding affinity (measured *in vitro*) is relatively low.

0.3 mg/kg BUP with 1.0 mg/kg NTX blocked drug-primed reinstatement to morphine- and cocaine-seeking. It is intuitive that the BUP/NTX combination should block reinstatement following a morphine prime, so long as the correct ratio and dose were selected. However, the mechanism by which the combination blocks

drug-primed reinstatement to cocaine is not clear, and the data presented here do not offer any answers. Following the same rationale as suggested for investigation of the aversive effects of the combination, BUP/NTX could be administered together with an NOP antagonist before presenting the drug prime. This might clarify if the NOP action of BUP contributes at all to the efficacy of the combination.

Interestingly, a large (300 patients, 11 sites) clinical study investigating the ability of BUP and NTX to reduce cocaine use has been initiated (design described in Mooney *et al.* 2013) despite the lack of knowledge of the pharmacological mechanisms involved. However, understanding the mechanism is likely to be important for optimising the therapy, and would certainly provide valuable insight to researchers designing novel single compound alternatives to BUP and NTX for use in relapse prevention (Kumar *et al*. 2014; Casal-Dominguez *et al.* 2014).

A final comment on the data presented in the first half of the thesis is that the similarity between the results for morphine-conditioned rats and cocaine-conditioned rats is not necessarily expected. Morphine and cocaine belong to very different classes of drug; they are a sedative and a stimulant respectively. The similarity observed counters the expectations of Tzschentke (2014) who stated that "when considering approaches to anti-abuse treatments, each drug class probably needs to be considered separately, as a drug that may be effective in supporting opioid abstinence would not necessarily be expected to reduce craving for cocaine as well".

In the second half of the thesis it was shown that **NTX is efficiently delivered by iontophoresis**. In fact, the concentration of NTX in the donor solution was reduced almost 400-fold from that first tested in order to match the requirements of this application. NTX's suitability for iontophoresis was largely expected considering its physicochemical properties.

The iontophoretic flux of BUP reported here is not superior to the passive flux of BUP from marketed passive patches. The rationale for iontophoresis of BUP was not to increase its flux compared to passive delivery, but to co-deliver it with NTX (to reduce abuse liability) and to increase control over input rates.

BUP accumulates in the skin, more so with higher pH and with higher BUP concentration in the donor. More BUP was recovered from the skin at pH 6 than at pH 4, implying that aqueous solubility is a bigger influence on accumulation than

electrostatic interaction with the skin (BUP is a weak base with pKa ~8). **The presence of BUP in the system retarded the convective solvent flow that normally boosts drug transport when delivering cations, and so reduced total flux of BUP and NTX.** This unusual effect has been observed previously for some small drug molecules and peptides, and can be 'managed' by keeping the concentration of the drug in the donor low.

Therapeutically relevant transdermal fluxes were observed for both BUP and NTX. However, the *in vitro* set-up does not mimic the presence of a functioning cutaneous blood supply, and therefore may not exactly predict *in vivo* transdermal fluxes. As NTX and BUP are both already marketed and known to be safe, it could be appropriate to go directly to tests in man (as opposed to *in vivo* testing in rodents, for example), for *in vivo* measurement of BUP and NTX plasma and stratum corneum concentrations. However, as BUP is a schedule 3 controlled drug, it is anticipated that it would be difficult to obtain consent for this work.

It is not known if a 24-hour-a-day iontophoresis system is feasible. None of the devices for iontophoretic delivery that have been licensed are intended for prolonged delivery. For example, dexamethasone administration is commonly performed for 20 minutes per session, and sumatriptan patches are only intended for up to 4 hours of use. The current density that can be tolerated for 24 hours is not clearly defined; Teillaud (1997) suggested 0.1 to 0.15 mA/cm^2 might be the maximum. However, skin response in healthy humans measured over a 24 hour period of iontophoresis (0.2 mA/cm^2 current density) was 'well tolerated' and only modest transient skin redness was observed (unpublished results by Maibach 1994, cited in Sage 1997). Ultimately, the maximum tolerable current density may depend on the nature of the electrode patch, for example on the type of hydrogel used (Nogueiras-Nieto *et al.* 2013). Furthermore, the practical consideration of battery life over longer periods of time may limit this application. A patch needing to be replaced more frequently than every 24 hours would lose one of the advantages of the transdermal route over oral tablets.

It was not the purpose of this thesis to compare the behaviour of a lipophilic and a hydrophilic weak base in a single transdermal iontophoresis system, however, NTX and BUP are similar in several physicochemical properties: the pKa of the two molecules are very similar, they are structurally related (see figure on page 21), and in the same size range, but differ in lipophilicity and water solubility. Therefore the observed differences in accumulation in skin, and in ability to alter the properties of

the skin, were presumed to be related to the differences in lipophilicity of the two drugs.

Practical implications

NTX is suited to iontophoretic delivery but BUP iontophoretic delivery may be more problematical. Two alternative transdermal options arise; firstly, development of a novel single compound alternative to BUP and NTX, with appropriate physicochemical properties for iontophoresis, or secondly, passive delivery of BUP with another mu antagonist with physicochemical properties more suited to passive delivery than NTX.

The findings of chapters 2 and 3 are useful regardless of the drug delivery route envisaged for patients. Whilst it is believed that the transdermal route offers genuine advantages for this combination, other drug delivery options such as an injectable depot, or sublingual or oral tablets should not be ruled out. BUP delivery by the oral route was rejected in favour of the sublingual route at least in part because low oral BUP bioavailability made this option costly (Sigmon *et al.* 2006). However, BUP became generic in 2009; perhaps BUP by the oral route could be re-examined. It is not yet known whether the far greater cost of a transdermal (iontophoretic) patch compared to an oral tablet is justifiable in terms of benefit to the patient.

If it is the lipophilicity of BUP that makes it accumulate in the skin during iontophoresis, and behave in an anomalous manner, that provides an interesting link between the two halves of the thesis; the relative lipophilicity of BUP has been said to have an important influence on its pharmacology (Lewis 1985) and on its pharmacokinetics (Robinson 2002).

Intriguingly, the NTX/BUP combination has also been proposed as a therapy for smoking cessation, alcohol dependence, weight loss and depression (McCann 2008; Almatroudi *et al*. 2015). The findings of chapter 2 and chapters 4 and 5 are equally applicable to these other therapeutic applications.

Overall, the combination holds great promise for relapse prevention, but the choice of delivery route remains open.

Conclusions

The mu-opioid receptor agonism of 0.3 mg/kg BUP was blocked by 1.0 mg/kg NTX, when administered via the intraperitoneal route in Sprague Dawley rats. At this delivery ratio, the combination was non-rewarding and non-aversive. Co-administration of 0.3 mg/kg BUP and 1.0 mg/kg NTX blocked drug-primed reinstatement to cocaine-seeking, and attenuated drug-primed reinstatement to morphine-seeking.

The transdermal iontophoretic flux of NTX was higher than the predicted target, whilst the flux of BUP was close to the predicted target. Varying the concentration of NTX in the donor solution when the concentration of BUP was fixed resulted in a predictable change in the flux of NTX, as explained by ion competition.

During iontophoresis a depot of BUP formed in the skin. The amount of drug recovered from the skin was proportional to the concentration of BUP in the donor and increased with pH. BUP caused a concentration-dependent decrease in anode-to-cathode EO, probably by neutralising negative charges on the skin. Auto-inhibition of BUP flux and a decreased flux of NTX was observed. This anomalous behaviour complicates optimisation of this drug combination, but iontophoresis still appears to be a feasible approach for delivering NTX and BUP through the skin.

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Appendix

	0.3 mg/kg BUP		0.3 mg/kg BUP & 0.3 mg/kg NTX		0.3 mg/kg BUP & 1.0 mg/kg NTX		0.3 mg/kg BUP & 3.0 mg/kg NTX		3.0 NTX mg/kg	
Replicate	Baseline	Post-conditioning	Baseline	Post-conditioning	Baseline	Post-conditioning	Baseline	Post-conditioning	Baseline	Post-conditioning
1	435	411	585	660	463	531	372	593	451	419
2	453	551	333	281	465	498	449	316	348	422
3	461	394	600	495	414	305	502	611	326	460
4	465	646	532	711	544	384	559	192	511	390
5	490	683	447	328	391	128	369	518	373	505
6	360	368	347	466	486	595	333	206	507	567
7	375	807	410	618	443	650	534	323	407	424
8	361	423			517	400	476	328		
9	505	506					429	451		
10	579	643					476	182		
11	508	660					492	650		
12	466	465					453	135		
13	439	542					456	263		
14	445	423					411	349		
15	367	632					435	170		
16							422	385		
Mean	447	544	465	508	465	436	448	355	418	455
SEM	16	34	41	62	18	60	15	42	28	23

Table A1: CPP data from chapter 2. Corrected time (seconds) in drug-paired compartment.

		Baseline			Post-conditioning			Extinction			Reinstatement		
Rat	DP compartment type	Squares	Circles	Centre	Squares	Circles	Centre	Squares	Circles	Centre	Squares	Circles	Centre
1	Squares	364	430	103	288	176	436	333	341	224	566	804	437
2	Circles	365	416	119	238	347	315	372	242	283	246	1053	499
3	Squares	448	332	119	531	234	134	246	478	174	910	678	207
4	Circles	295	448	157	354	311	234	125	310	365	323	1017	460
5	Circles	402	387	102	254	519	126	293	455	152	540	1097	162
6	Squares	411	365	124	364	445	91	376	356	168	526	824	449
7	Circles	343	379	177	325	318	254	200	420	276	351	974	473
8	Squares	392	348	159	334	327	239	266	333	302	397	1089	292
9	Squares	276	396	213	454	209	235	408	271	236	408	980	408
10	Circles	312	494	94	214	504	181	215	165	519	291	1358	150
11	Squares	468	265	166	399	246	252	299	310	287	526	746	524
12	Circles	468	248	183	246	552	98	279	329	293	376	904	517
13	Circles	255	529	116	537	210	148	150	299	450	541	479	767
14	Squares	262	243	393	449	283	165	368	274	250	1307	238	255
15	Circles	446	329	123	271	340	287	291	398	208	934	540	323
16	Squares	322	386	191	233	386	280	409	313	178	453	747	599
17	Circles	380	270	245	161	221	518	282	403	223	347	561	876
18	Squares	337	451	113	326	399	175	458	310	132	848	766	183
19	Circles	369	368	161	307	403	189	248	444	207	601	1022	134
20	Squares	293	430	177	396	368	136	470	265	166	987	607	207
Mean		360	376	162	334	340	225	304	336	255	574	824	396
SEM		15	18	15	23	24	24	21	18	22	63	59	46

Table A2: Example of CPP raw data from chapter 3. Time (seconds) in each of the three compartments. Cocaine drug-prime control.

Replicate	Baseline	Post-conditioning	Extinction	Reinstatement
1	413	559	445	372
2	480	534	355	730
3	517	625	306	516
4	441	604	547	603
5	369	616	541	265
6	551	632	390	741
7	312	623	487	636
8	467	552	516	761
9	382	501	520	330
10	374	521	529	556
11	449	511	577	567
12	365	466	575	557
Mean	427	562	482	553
SEM	20	16	26	47

Table A3: CPP data from chapter 3. Control cocaine drug-prime. Corrected time (seconds) in drug-paired compartment.

Replicate	Baseline	Post-conditioning	Extinction	Reinstatement
1	560	616	597	514
2	450	542	496	385
3	504	570	590	566
4	436	531	279	511
5	539	624	495	149
6	329	363	381	427
7	430	710	709	385
8	333	364	353	131
9	460	606	194	212
Mean	449	547	455	365
SEM	27	39	13	13

Table A4: CPP data from chapter 3. Blocking of cocaine drug-prime with BUP/NTX. Corrected time (seconds) in drug-paired compartment.

Replicate	Baseline	Post-conditioning	Extinction	Reinstatement
1	304	543	328	651
2	517	617	591	620
3	437	635	328	445
4	463	759	550	371
5	460	519	583	654
6	437	655	470	640
7	475	608	699	865
8	356	516	446	858
9	471	628	481	571
10	493	669	375	643
11	340	634	630	300
12	507	580	384	731
13	432	600	655	814
14	337	495	229	187
Mean	431	604	482	596
SEM	18	19	38	55

Table A5: CPP data from chapter 3. Control morphine drug-prime. Corrected time (seconds) in drug-paired compartment.

Replicate	Baseline	Post-conditioning	Extinction	Reinstatement
1	336	554	447	633
2	377	615	531	654
3	304	626	319	128
4	531	692	476	470
5	335	675	278	115
6	476	572	301	791
7	374	497	355	485
8	434	645	664	740
Mean	396	610	421	502
SEM	28	23	47	92

Table A6: CPP data from chapter 3. Blocking of morphine drug-prime with BUP/NTX. Corrected time (seconds) in drug-paired compartment.

Data from chapter 4

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
3	0.15	0.00	0.00	0.00	0.04	0.08
6	0.09	0.08	0.01	0.03	0.05	0.04

Table A7: Passive flux of BUP (µg/h) from 0.55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
3	0.00	0.00	0.00	0.00	0.0	0.0
6	0.09	0.06	0.00	0.00	0.04	0.04

Table A8: Passive flux of NTX (µg/h) from 0.55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.11	0.08	0.00	0.00	0.05	0.06
4	0.14	0.13	0.00	0.00	0.07	0.08
5	0.17	0.22	0.09	0.13	0.15	0.06
6	0.29	0.33	0.12	0.15	0.22	0.10

Table A9: Iontophoretic flux of BUP (µg/h) from 55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	21	46	22	35	31	12
2	111	170	106	140	132	29
3	253	287	220	242	251	28
4	299	269	274	271	278	14
5	294	248	249	249	260	23
6	322	272	219	275	272	42

Table A10: Iontophoretic flux of NTX (µg/h) from 55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.04	0.00	0.01	0.02
3	0.04	0.04	0.16	0.01	0.06	0.07
4	0.11	0.09	0.32	0.04	0.14	0.12
5	0.37	0.18	0.42	0.09	0.26	0.16
6	0.37	0.34	0.67	0.14	0.38	0.22

Table A11: Iontophoretic flux of BUP (µg/h) from 5.5:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	1	3	9	1	3	4
2	33	30	67	13	36	23
3	68	71	103	29	68	31
4	92	78	127	41	85	36
5	181	101	121	47	113	56
6	117	136	147	50	113	43

Table A12: Iontophoretic flux of NTX (µg/h) from 5.5:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Cell 10	Mean	SD
1	0.40	0.02	0.06	0.07	0.08	0.08	0.17	1.09	0.21	0.15	0.23	0.32
2	1.18	0.34	0.19	0.02	0.05	0.01	0.08	1.92	0.11	0.05	0.39	0.64
3	2.51	0.99	0.87	0.13	0.33	0.15	0.15	2.48	0.36	0.25	0.82	0.93
4	3.00	1.07	1.14	0.49	0.55	0.39	0.31	2.83	0.75	0.52	1.11	0.99
5	4.35	2.37	1.70	1.17	0.87	0.63	0.52	3.29	1.15	0.90	1.70	1.27
6	4.83	2.62	2.10	1.27	1.33	0.83	0.97	3.60	1.54	1.36	2.04	1.29

Table A13: Table 10: Iontophoretic flux BUP (µg/h) from 0.55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Cell 10	Mean	SD
1	15	9	4	2	2	2	1	5	2	2	4	4
2	21	18	11	10	9	8	8	12	8	9	11	5
3	26	26	21	12	17	15	13	17	14	16	18	5
4	24	23	19	17	18	16	18	19	17	19	19	3
5	30	26	24	30	21	18	22	23	20	23	24	4
6	24	26	23	19	24	20	25	26	22	29	24	3

Table A14: Iontophoretic flux of NTX (µg/h) from 0.55:1 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.14	0.03	0.07
2	0.06	0.21	0.05	0.77	0.27	0.34
3	0.39	0.58	0.33	1.69	0.75	0.64
4	1.66	1.23	1.28	2.47	1.66	0.57
5	1.46	1.62	0.97	2.75	1.70	0.75
6	1.57	1.89	1.28	2.84	1.90	0.68

Table A15: Iontophoretic flux of BUP (µg/h) from 1:0 (ratio NTX:BUP)

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.13	0.14	0.08	0.14	0.39	0.26	0.19	0.11
3	0.36	0.35	0.23	0.48	0.77	0.44	0.44	0.18
4	0.55	0.37	0.33	0.65	1.17	0.68	0.63	0.30
5	0.74	0.49	0.41	0.70	1.68	0.78	0.80	0.46
6	0.85	0.56	0.51	0.62	1.69	0.82	0.84	0.44

Table A16: Fluxes (µg/h) of acetaminophen. Donor and receiver of pH 4.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.40	0.22	0.34	0.96	0.90	0.94	0.63	0.34
3	1.43	1.01	1.22	2.12	2.00	2.04	1.64	0.48
4	2.31	1.67	1.82	2.87	2.76	3.10	2.42	0.58
5	2.98	2.15	2.30	3.08	3.56	3.64	2.95	0.62
6	3.15	2.64	2.68	2.56	3.49	3.62	3.02	0.46

Table A17: Fluxes (µg/h) of naltrexone. Donor and receiver of pH 4.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.34	0.06	0.14
3	0.15	0.00	0.00	0.32	0.00	1.12	0.27	0.44
4	0.52	0.00	0.00	1.37	1.20	2.46	0.93	0.95
5	1.35	0.13	0.46	2.45	2.56	3.50	1.74	1.32
6	2.36	0.42	0.87	2.87	4.10	5.16	2.63	1.83

Table A18: Fluxes (µg/h) of buprenorphine. Donor and receiver of pH 4.

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.10	4.20	1.47	2.67	0.63	0.89	2.20	1.44	1.92	2.36	2.26	2.19
2	2.24	1.65	1.96	2.19	1.02	0.84	2.96	0.83	3.04	0.89	2.92	2.06
3	2.34	1.80	2.27	1.80	2.14	2.05	3.64	0.68	3.55	0.80	3.37	1.56
4	2.60	1.73	2.42	1.40	2.48	1.64	4.00	0.43	3.98	0.72	3.74	1.83
5	2.79	1.98	2.42	1.12	3.25	1.61	4.33	0.49	4.33	0.72	4.00	1.26
6	2.79	1.42	2.42	1.26	3.40	1.38	4.58	0.38	4.89	1.01	4.22	1.29
7	2.88	2.35	2.47	1.06	3.49	1.29	4.91	0.46	5.33	1.28	4.50	0.94
8	2.88	1.59	2.50	0.91	3.66	3.86	5.21	0.32	5.78	0.86	4.76	0.99
9	2.88	1.12	2.50	1.21	3.83	1.76	5.61	0.88	6.06	0.80	5.17	1.01
10	2.92	1.60	2.50	0.78	3.86	1.39	5.75	0.37	6.40	0.59	5.40	0.70
11	2.92	1.43	2.50	0.95	3.94	2.02	5.95	0.12	6.62	0.73	5.70	0.88
12	2.92	1.06	2.50	1.07	4.19	1.45	6.26	0.14	6.83	0.49	6.00	0.89
Total mass (µg)		21.9		16.4		20.2		6.5		11.2		15.6

Table A19: BUP in tapes. Donor and receiver of pH 4.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.17	0.10	0.07	0.26	0.00	0.13	0.12	0.09
3	0.40	0.29	0.17	0.58	0.17	0.33	0.32	0.15
4	0.69	0.51	0.35	0.89	0.30	0.57	0.55	0.22
5	0.77	0.68	0.41	1.08	0.47	0.80	0.70	0.24
6	0.83	0.83	0.48	1.17	0.57	0.82	0.78	0.24

Table A20: Fluxes (µg/h) of acetaminophen. Donor and receiver of pH 5.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.64	0.15	0.13	0.63	0.00	0.00	0.26	0.30
3	1.59	0.72	0.55	1.72	0.00	0.69	0.88	0.66
4	2.63	1.53	1.29	2.81	1.03	1.30	1.76	0.76
5	3.49	2.35	1.78	3.38	1.83	2.20	2.51	0.75
6	3.81	2.66	1.90	3.49	2.28	2.63	2.79	0.72

Table A21: Fluxes (µg/h) of naltrexone. Donor and receiver of pH 5.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.56	0.00	0.00	0.55	0.00	0.00	0.18	0.29
5	1.51	0.00	0.00	1.94	0.00	0.46	0.65	0.86
6	2.38	1.08	0.64	2.88	0.60	0.76	1.39	0.99

Table A22: Fluxes (µg/h) of buprenorphine. Donor and receiver of pH 5.

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.0	9.2	1.6	3.7	1.1	5.4	0.51	7.9	2.2	7.7	0.8	2.1
2	3.0	4.8	2.4	2.7	1.6	2.7	0.5	2.8	3.0	3.5	1.4	1.7
3	3.7	4.3	2.8	1.6	1.6	1.6	0.5	1.4	3.7	2.4	1.9	1.4
4	4.5	2.3	3.2	1.9	1.7	1.6	0.5	1.0	4.8	2.4	2.5	1.6
5	5.1	2.2	3.6	2.0	1.7	1.6	0.6	1.1	5.3	2.1	3.1	2.4
6	5.5	1.8	3.9	1.4	1.9	1.6	0.9	1.1	5.6	1.5	3.8	2.8
7	5.8	1.9	4.2	1.4	2.1	1.2	0.9	1.0	6.0	2.0	4.7	1.9
8	6.3	2.3	4.4	1.2	2.2	1.0	1.0	0.6	6.3	1.1	5.2	1.7
9	6.7	1.3	4.7	1.2	2.2	1.3	1.1	0.6	6.6	1.3	5.7	1.1
10	7.1	1.5	5.0	1.1	2.3	0.5	1.1	0.4	6.9	0.6	6.2	2.0
11	7.4	1.2	5.4	1.2	2.3	0.5	1.1	0.8	7.2	1.2	6.6	1.2
12	7.7	0.8	5.6	1.1	2.5	0.0	1.2	0.6	7.4	1.0	6.8	0.8
Total mass (µg)		33.5		20.7		18.9		19.4		26.8		20.7

Table A23: BUP in tapes. Donor and receiver of pH 5.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.22	0.42	0.18	0.16	0.21	0.35	0.26	0.11
3	0.47	0.85	0.47	0.45	0.55	0.89	0.61	0.20
4	0.74	1.59	0.70	0.62	0.69	1.06	0.90	0.37
5	0.87	1.11	0.93	0.71	1.17	1.36	1.03	0.23
6	0.86	1.45	0.97	0.76	1.02	1.51	1.10	0.31

Table A24: Fluxes (µg/h) of acetaminophen. Donor and receiver of pH 6.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.47	0.83	0.40	0.57	0.51	0.53	0.55	0.15
3	1.49	1.92	1.15	0.46	1.60	1.60	1.37	0.51
4	2.40	4.04	2.08	2.42	2.17	2.08	2.53	0.75
5	2.87	2.60	2.81	2.67	2.72	2.60	2.71	0.11
6	3.04	3.75	2.88	3.24	3.63	3.29	3.31	0.33

Table A25: Fluxes (µg/h) of naltrexone. Donor and receiver of pH 6.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.39	0.29	0.00	0.11	0.18
4	0.67	2.33	0.41	0.92	0.81	0.45	0.93	0.71
5	0.65	2.36	0.60	1.78	1.84	1.00	1.37	0.72
6	1.01	4.21	0.54	2.79	3.54	2.02	2.36	1.43

Table A26: Fluxes (µg/h) of buprenorphine. Donor and receiver of pH 6.

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	3.0	142.9	1.8	15.2	2.8	136.9	1.0	17.0	1.5	7.7	2.6	13.5
2	3.9	13.6	2.8	6.3	3.7	17.9	1.7	13.0	2.1	0.3	3.6	4.7
3	4.4	3.0	3.5	3.9	4.4	10.8	2.1	2.0	2.4	0.4	4.4	3.3
4	4.9	4.5	4.0	2.0	4.9	8.0	2.6	2.2	2.9	0.5	5.1	3.3
5	5.1	1.6	4.4	1.5	5.3	5.2	3.1	2.1	3.1	1.2	6.0	2.6
6	5.6	3.2	4.7	1.1	5.5	2.4	3.5	1.5	3.5	1.5	6.9	2.9
7	6.0	1.6	5.4	1.0	5.9	2.3	3.7	0.9	3.8	1.2	7.7	2.6
8	6.2	1.4	5.7	0.7	6.2	3.4	4.1	1.8	4.5	2.2	8.6	2.0
9	6.5	1.5	6.1	1.0	6.6	3.6	4.5	1.0	4.9	1.3	9.3	1.0
10	6.7	0.9	6.4	0.5	6.9	1.6	4.7	0.8	5.1	0.8	10.0	0.9
11	6.9	0.9	6.7	0.4	7.2	1.2	5.0	0.7	5.3	0.7	10.8	1.3
12	7.1	1.0	6.9	0.4	7.5	1.7	5.2	0.8	5.4	0.6	11.3	1.2
Total mass (µg)		176.3		33.8		194.9		43.9		18.3		39.4

Table A27: BUP in tapes. Donor and receiver of pH 6.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.06	0.00	0.11	0.00	0.04	0.05
2	0.53	0.23	0.56	0.27	0.40	0.17
3	1.19	0.55	1.19	0.70	0.91	0.33
4	1.68	0.94	1.67	1.12	1.35	0.38
5	1.98	1.13	1.77	1.44	1.58	0.37
6	1.99	1.38	2.00	1.68	1.76	0.29

Table A28: Fluxes (µg/h) of acetaminophen. Donor and receiver of pH 6, no BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.85	0.53	0.85	1.10	0.83	0.23
3	2.03	1.77	2.08	2.41	2.07	0.26
4	3.22	2.98	3.23	3.52	3.24	0.22
5	4.01	3.70	3.73	4.36	3.95	0.31
6	3.73	3.58	4.15	4.43	3.97	0.39

Table A29: Fluxes (µg/h) of naltrexone. Donor and receiver of pH 6, no BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.12	0.37	0.22	0.11	0.21	0.12
3	0.33	0.61	0.41	0.35	0.42	0.13
4	0.60	0.71	0.77	0.47	0.64	0.13
5	0.73	0.84	0.92	0.70	0.80	0.10
6	0.91	0.82	1.14	0.84	0.93	0.15

Table A30: Fluxes (µg/h) of acetaminophen. 0.14 & 0.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.43	0.37	0.00	0.20	0.23
2	0.86	1.74	1.19	1.02	1.20	0.38
3	1.93	2.81	2.31	2.08	2.28	0.39
4	2.79	3.05	3.08	2.60	2.88	0.23
5	3.54	3.68	3.52	3.55	3.57	0.07
6	4.04	3.17	3.67	3.44	3.58	0.37

Table A31: Fluxes (µg/h) of naltrexone. 0.14 & 0.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.72	0.00	0.00	0.18	0.36
2	0.30	1.38	0.14	0.00	0.46	0.63
3	0.80	3.05	0.60	0.42	1.22	1.23
4	1.49	4.57	1.32	0.97	2.08	1.67
5	2.45	6.21	2.02	1.69	3.09	2.11
6	3.02	5.96	2.85	2.21	3.51	1.67

Table A32: Fluxes (µg/h) of buprenorphine. 0.14 & 0.5 mg/ml NTX & BUP.

	Cell 1		Cell 2		Cell 3		Cell 4	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.4	3.4	1.6	1.19	2.5	2.5	3.7	2.9
2	3.3	1.64	2.4	0.74	3.3	1.11	4.4	0.63
3	4.4	1.35	3.2	0.49	3.8	0.83	5.0	0.63
4	5.2	0.62	3.5	0.18	4.2	0.9	5.4	0.6
5	5.6	0.47	3.9	0.48	4.5	0.6	5.6	0.5
6	6.0	0.47	4.3	0.31	4.6	0.6	5.7	0.4
7	6.4	0.40	4.6	0.40	4.8	0.5	6.0	0.4
8	6.8	0.18	5.1	0.62	5.3	0.8	6.1	0.4
9	7.1	0.36	5.3	0.74	5.5	0.6	6.3	0.5
10	7.3	0.29	5.7	0.69	6.0	0.82	6.6	0.03
11	7.7	0.34	6.0	1.28	6.3	0.53	6.7	0.24
12	8.1	0.31	6.2	0.16	6.6	0.47	6.9	0.41
Total mass in tapes (µg)		9.8		7.3		10.3		7.6
BUP in viable tissue (µg)		25.4		32.6		24.0		29.7
NTX in viable tissue (µg)		7.2		4.7		6.7		8.1

Table A33: Drug in the skin. 0.14 & 0.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.30	0.13	0.05	0.12	0.13
3	0.14	0.41	0.23	0.10	0.22	0.14
4	0.21	0.42	0.32	0.15	0.28	0.12
5	0.38	0.52	0.56	0.32	0.45	0.12
6	0.36	0.40	0.40	0.31	0.37	0.04

Table A34: Fluxes (µg/h) of acetaminophen. 0.14 & 2.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.29	0.83	0.86	0.57	0.64	0.27
3	1.11	1.84	1.74	1.53	1.55	0.32
4	1.97	2.48	2.20	2.28	2.23	0.21
5	2.65	2.74	3.06	2.57	2.76	0.22
6	2.34	2.68	2.08	2.23	2.33	0.26

Table A35: Fluxes (µg/h) of naltrexone. 0.14 & 2.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.45	0.43	0.00	0.22	0.25
3	0.15	2.57	0.87	0.33	0.98	1.10
4	0.51	4.45	1.88	1.05	1.98	1.74
5	1.52	5.45	3.85	1.98	3.20	1.81
6	2.01	5.74	5.43	2.47	3.91	1.94

Table A36: Fluxes (µg/h) of buprenorphine. 0.14 & 2.5 mg/ml NTX & BUP.

	Cell 1		Cell 2		Cell 3		Cell 4	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	3.6	94.1	2.0	38.4	3.1	58.1	3.7	101.5
2	5.3	33.4	2.6	19.71	4.3	28.64	5.2	36.84
3	6.2	15.1	3.3	11.43	4.9	10.13	6.0	17.04
4	7.1	11.9	4.0	10.64	5.5	16.7	6.8	12.3
5	7.4	7.5	4.7	10.57	6.4	19.1	7.5	11.7
6	7.9	7.3	5.3	9.83	7.0	9.0	8.0	6.7
7	8.4	5.5	5.8	6.85	7.5	6.3	8.6	8.6
8	8.7	5.1	6.2	4.19	7.9	5.4	9.0	4.0
9	8.9	3.3	6.6	0.52	8.4	5.0	9.5	3.4
10	9.4	4.0	6.9	0.33	8.8	6.56	9.8	2.70
11	9.7	5.4	7.3	0.37	9.2	3.86	10.4	2.67
12	9.9	2.5	7.7	0.45	9.6	3.04	10.9	3.34
Total mass in tapes (µg)		194.9		113.3		171.8		210.6
BUP in viable tissue (µg)		123.0		147.8		132.5		119.3
NTX in viable tissue (µg)		6.0		3.9		4.5		4.6

Table A37: Drug in the skin. 0.14 & 2.5 mg/ml NTX & BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.24	0.00	0.32	0.25	0.20	0.14
3	0.69	0.55	0.60	0.49	0.58	0.08
4	1.34	0.87	1.26	0.44	0.98	0.41
5	1.23	1.00	0.95	0.93	1.03	0.14
6	1.44	1.23	1.27	0.69	1.16	0.32

Table A38: Fluxes (μg/h) of acetaminophen. Low current density.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.51	0.31	0.21	0.25
3	0.44	0.73	3.05	0.95	1.29	1.19
4	1.31	1.48	1.73	0.94	1.37	0.34
5	1.58	1.69	2.57	1.95	1.95	0.44
6	2.30	2.51	3.66	1.72	2.55	0.81

Table A39: Fluxes (μg/h) of naltrexone. Low current density.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.33	0.47	1.53	0.00	0.58	0.66
5	0.22	1.07	2.45	1.69	1.36	0.95
6	0.82	1.71	2.59	1.77	1.72	0.72

Table A40: Fluxes (μg/h) of buprenorphine. Low current density.

	Cell 1		Cell 2		Cell 3		Cell 4	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.2	2.4	1.7	2.2	2.7	5.8	3.8	70.9
2	3.3	0.72	2.5	0.52	3.3	1.21	4.8	5.59
3	4.3	0.78	3.3	0.37	3.9	0.57	5.3	2.19
4	4.9	0.41	4.0	0.2	4.7	0.79	5.9	1.7
5	5.8	0.4	4.9	0.3	5.1	0.51	6.7	1.4
6	6.2	0.3	5.8	0.2	5.6	0.64	7.3	0.9
7	7.2	0.5	6.7	0.2	5.9	0.42	7.9	1.0
8	7.8	0.3	7.5	0.1	6.4	0.41	8.4	0.8
9	8.4	0.2	8.4	0.2	7.7	0.25	8.7	0.7
10	9.0	0.13	9.3	0.22	8.2	0.29	9.3	0.49
11	9.5	0.15	10.0	0.17	8.7	0.30	9.8	0.26
12	10.0	0.17	10.6	0.19	9.2	0.43	10.3	0.52
Total mass in tapes (µg)		6.5		4.9		11.6		86.5

Table A41: BUP in tapes. Low current density.

Data from chapter 5

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.11	0.05	0.06	0.00	0.00	0.00	0.04	0.05
3	0.28	0.16	0.23	0.21	0.22	0.26	0.23	0.04
4	0.45	0.29	0.40	0.29	0.35	0.46	0.37	0.08
5	0.54	0.33	0.51	0.48	0.51	0.68	0.51	0.11
6	0.34	0.58	0.56	0.47	0.53	0.58	0.51	0.09

Table A42: Fluxes (µg/h) of acetaminophen. Receiver solution B (PBS pH 7.4).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.36	0.18	0.14	0.00	0.00	0.00	0.11	0.14
3	1.16	0.67	0.74	0.49	0.49	1.05	0.77	0.28
4	2.19	1.50	1.55	1.24	1.12	2.08	1.61	0.44
5	2.89	1.52	2.14	1.96	1.86	2.75	2.19	0.53
6	1.71	2.76	2.50	1.99	2.06	2.71	2.29	0.43

Table A43: Fluxes (µg/h) of naltrexone. Receiver solution B (PBS pH 7.4).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.12	0.00	0.42	0.00	0.00	0.00	0.09	0.17
3	0.33	0.12	0.58	0.00	0.00	0.00	0.17	0.24
4	0.48	0.00	0.63	0.27	0.00	0.00	0.23	0.28
5	0.48	0.00	0.60	0.23	0.00	0.00	0.22	0.27
6	0.57	0.00	0.35	0.32	0.00	0.00	0.21	0.24

Table A44: Fluxes (µg/h) of buprenorphine. Receiver solution B (PBS pH 7.4).

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.4	7.8	2.6	10.2	2.2	5.3	2.3	7.4	2.3	11.3	2.4	4.4
2	3.2	2.5	3.2	3.5	2.7	2.6	3.1	4.4	3.2	3.5	3.4	2.9
3	3.8	2.6	3.7	3.6	3.3	2.3	3.9	2.8	4.1	2.3	4.0	1.3
4	4.3	1.7	4.3	2.3	3.8	1.8	4.3	1.8	4.8	1.8	4.5	1.0
5	4.7	1.4	4.3	1.8	4.2	1.3	4.8	1.8	5.2	1.3	5.3	0.7
6	5.1	1.6	4.6	1.6	4.6	1.3	5.2	1.4	5.7	1.0	5.8	1.0
7	5.3	1.1	4.9	1.5	5.1	2.0	5.6	1.5	6.2	1.1	6.3	0.7
8	5.6	0.6	5.1	1.1	5.4	1.0	5.9	1.4	6.5	2.6	6.7	1.1
9	5.8	0.7	5.6	1.4	5.8	1.4	6.2	1.0	7.1	0.9	7.2	0.6
10	6.2	1.3	5.9	0.8	6.2	1.0	6.8	1.2	7.5	0.8	7.9	0.5
11	6.3	0.5	6.3	1.4	6.3	0.9	7.1	0.8	8.0	0.6	8.7	0.3
12	6.7	0.8	6.6	1.2	6.7	1.5	7.4	0.9	8.3	0.5	9.1	0.4
Total mass (µg)		22.6		30.4		22.4		26.4		27.7		14.9

Table A45: BUP in tapes. Receiver solution B (PBS pH 7.4).

Hours	Cell 1	Cell 2
1	0.00	0.00
2	0.09	0.30
3	0.26	0.58
4	0.40	0.70
5	0.33	0.81
6	0.38	0.71

Table A46: Fluxes (µg/h) of acetaminophen. Receiver solution C (Tris pH 7.4).

Hours	Cell 1	Cell 2
1	0.00	0.00
2	0.00	0.71
3	0.73	1.63
4	1.29	2.27
5	1.35	3.02
6	1.67	3.18

Table A47: Fluxes (µg/h) of naltrexone. Receiver solution C (Tris pH 7.4).

Hours	Cell 1	Cell 2
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.00	0.00
6	0.00	0.32

Table A48: Fluxes (µg/h) of buprenorphine. Receiver solution C (Tris pH 7.4).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.28	0.15	0.20	0.61	0.00	0.25	0.23
3	0.63	0.46	0.59	1.14	0.12	0.59	0.37
4	0.98	0.76	1.05	1.44	0.27	0.90	0.43
5	1.42	1.02	1.69	1.91	0.48	1.30	0.57
6	1.59	1.25	2.01	2.07	0.61	1.51	0.60

Table A49: Fluxes (µg/h) of acetaminophen. Receiver solution D (phosphate buffer pH 7.4 no chloride).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.53	0.51	0.72	2.05	0.00	0.76	0.77
3	2.13	1.96	2.60	4.39	0.28	2.27	1.47
4	3.64	3.40	4.18	5.79	0.94	3.59	1.75
5	5.20	4.44	5.35	6.91	1.57	4.70	1.96
6	5.45	5.21	6.09	6.97	2.06	5.16	1.86

Table A50: Fluxes (µg/h) of naltrexone. Receiver solution D (phosphate buffer pH 7.4 no chloride).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.32	0.37	0.00	0.00	0.00	0.14	0.19

Table A51: Fluxes (µg/h) of buprenorphine. Receiver solution D (phosphate buffer pH 7.4 no chloride).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.09	0.30	0.34	0.41	0.28	0.14
3	0.27	0.61	0.56	0.72	0.54	0.19
4	0.48	0.74	0.67	0.89	0.70	0.17
5	0.60	0.99	0.71	0.89	0.80	0.17
6	0.51	0.90	0.70	0.92	0.76	0.19

Table A52: Fluxes (µg/h) of acetaminophen. Receiver solution E (NaCl only).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.25	0.63	1.26	0.83	0.44	0.27
3	1.06	1.35	2.23	1.95	1.20	0.20
4	2.07	1.63	3.00	2.78	1.85	0.32
5	2.78	2.45	3.14	2.64	2.62	0.23
6	2.21	2.35	3.04	2.88	2.28	0.10

Table A53: Fluxes (µg/h) of naltrexone. Receiver solution E (NaCl only).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.93	0.62	0.39	0.47
4	0.00	0.00	2.25	1.52	0.94	1.13
5	0.00	0.72	3.26	2.27	1.56	1.48
6	0.20	0.91	4.25	2.73	2.02	1.83

Table A54: Fluxes (µg/h) of buprenorphine. Receiver solution E (NaCl only).

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.16	0.11	0.10	0.00	0.12	0.20	0.12	0.07
3	0.29	0.25	0.28	0.17	0.28	0.43	0.28	0.09
4	0.39	0.34	0.39	0.24	0.44	0.52	0.39	0.09
5	0.42	0.60	0.50	0.38	0.49	0.65	0.51	0.10
6	0.48	0.61	0.56	0.37	0.56	0.53	0.52	0.08
7	0.53	0.63	0.61	0.54	0.75	0.63	0.61	0.08
8	0.43	0.51	0.45	0.45	0.57	0.51	0.49	0.05
9	0.34	0.36	0.35	0.39	0.50	0.42	0.39	0.06
23	0.11	0.07	0.12	0.11	0.15	0.12	0.11	0.02
24	0.05	0.11	0.06	0.11	0.09	0.10	0.09	0.03

Table A55: Fluxes (µg/h) of acetaminophen. Post-iontophoretic release.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.43	0.59	0.31	0.26	0.36	0.43	0.40	0.11
3	1.13	1.55	1.18	0.94	1.14	1.46	1.23	0.23
4	1.81	2.30	1.75	1.37	1.65	1.90	1.80	0.31
5	2.16	3.44	2.27	1.86	2.15	2.42	2.38	0.55
6	2.52	3.47	2.63	2.32	2.33	2.18	2.57	0.47
7	2.50	2.94	2.42	2.45	2.53	2.39	2.54	0.20
8	1.77	2.19	1.80	1.82	1.87	1.82	1.88	0.15
9	1.41	1.53	1.46	1.56	1.62	1.51	1.51	0.08
23	0.49	0.41	0.48	0.56	0.57	0.46	0.49	0.06
24	0.12	0.05	-0.01	0.15	0.20	0.26	0.13	0.10

Table A56: Fluxes (µg/h) of naltrexone. Post-iontophoretic release.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.17	0.11	0.12	0.00	0.00	0.00	0.07	0.08
5	0.34	0.36	0.25	0.00	0.00	0.27	0.20	0.16
6	0.49	0.60	0.49	0.14	0.29	0.26	0.38	0.17
7	1.16	1.44	1.26	0.55	0.82	1.02	1.04	0.32
8	1.32	1.63	1.35	0.53	0.80	1.04	1.11	0.40
9	1.56	1.89	1.57	0.69	0.97	1.43	1.35	0.44
23	1.42	1.77	1.65	0.88	1.01	1.35	1.35	0.35
24	1.87	1.83	1.68	1.26	1.01	1.66	1.55	0.34

Table A57: Fluxes (µg/h) of buprenorphine. Post-iontophoretic release.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.00	0.00	0.14	0.05	0.08
2	0.13	0.00	0.30	0.14	0.15
3	0.13	0.23	0.45	0.27	0.16
4	0.21	0.25	0.51	0.32	0.16
5	0.31	0.30	0.49	0.36	0.11
6	0.33	0.39	0.46	0.39	0.06
7	0.47	0.54	0.56	0.52	0.05
8	0.68	0.60	0.65	0.64	0.04

Table A58: Fluxes (μg/h) of acetaminophen. 3 compartment control.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.20	0.17	0.28	0.22	0.05
2	0.70	1.03	1.35	1.03	0.33
3	1.52	1.95	2.28	1.92	0.38
4	2.28	2.75	2.66	2.56	0.25
5	2.74	3.02	2.79	2.85	0.15
6	2.99	3.56	3.36	3.30	0.29
7	3.44	3.60	2.97	3.34	0.33
8	3.97	4.05	4.13	4.05	0.08

Table A59: Fluxes (μg/h) of naltrexone. 3 compartment control.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.21	0.07	0.12
3	0.00	0.00	0.55	0.18	0.32
4	0.00	0.25	0.72	0.32	0.36
5	0.38	0.56	1.00	0.65	0.32
6	0.47	0.75	1.05	0.76	0.29
7	0.59	1.10	1.00	0.90	0.27
8	0.90	1.38	1.52	1.27	0.33

Table A60: Fluxes (μg/h) of buprenorphine. 3 compartment control.

	Cell 1		Cell 2		Cell 3	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.3	6.1	3.5	8.6	2.3	5.8
2	4.4	4.0	4.5	3.2	3.1	2.4
3-5	6.4	9.3	5.7	6.3	4.3	4.3
6-8	9.3	6.0	7.6	4.4	6.3	3.7
9-12	11.1	4.3	8.9	2.7	7.4	2.4
Total BUP in tapes (µg)		29.7		25.2		18.6
BUP in viable tissue (µg)		36.2		32.6		50.0
NTX in viable tissue (µg)		7.8		7.9		9.0

Table A61: Drug in the skin. 3 compartment control.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.00	0.09	0.00	0.03	0.05
2	0.00	0.00	0.00	0.00	0.00
3	0.14	0.12	0.00	0.09	0.08
4	0.11	0.09	0.00	0.07	0.06
5	0.07	0.04	0.11	0.08	0.03
6	0.20	0.14	0.08	0.14	0.06
7	0.25	0.16	0.11	0.17	0.07
8	0.16	0.11	0.11	0.12	0.03

Table A62: Fluxes (µg/h) of acetaminophen. Reversing polarity.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.00	0.00	0.00	0.00	0.00
2	0.81	0.79	0.25	0.62	0.32
3	1.35	1.37	0.77	1.16	0.34
4	1.60	1.16	1.26	1.34	0.23
5	2.01	1.56	1.72	1.77	0.23
6	2.46	2.32	1.78	2.19	0.36
7	2.34	2.32	1.74	2.13	0.34
8	2.65	2.39	2.58	2.54	0.14

Table A63: Fluxes (µg/h) of naltrexone. Reversing polarity.

Hours	Cell 1	Cell 2	Cell 3	Mean	SD
1	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
5	0.00	0.21	0.16	0.12	0.11
6	0.27	0.31	0.10	0.23	0.11
7	0.27	0.35	0.31	0.31	0.04
8	0.38	0.39	0.22	0.33	0.10

Table A64: Fluxes (µg/h) of buprenorphine. Reversing polarity.

	Cell 1				Cell 2				Cell 3	
	+/-		-/+		+/-		-/+		+/-	-/+
Tape/s	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Mass of BUP (µg)	Mass of BUP (µg)
1	2.1	1.7	3.5	9.8	2.9	4.8	3.6	8.8	4.1	7.1
2	5.3	3.9	4.7	3.6	3.7	1.2	4.7	2.4	1.4	2.3
3-5	7.3	4.1	5.8	4.1	4.8	1.3	5.6	4.0	2.0	3.9
6-8	9.1	1.9	6.8	2.1	5.7	0.9	6.7	2.4	1.3	2.5
9-12	10.6	1.2	7.9	1.9	6.8	0.8	7.2	1.5	0.8	2.1
Total BUP in tapes (µg)		12.8		21.5		8.9		19.1	9.6	18.0
BUP in viable tissue (µg)		15.2		19.1		10.7		17.8	9.8	17.2
NTX in viable tissue (µg)		1.5		5.2		1.9		4.8	3.6	4.3

Table A65: Drug in the skin. Reversing polarity. No tape weights available for cell 3.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.09	0.21	0.18	0.47	0.14	0.20	0.16	0.15
3	0.08	0.12	0.35	0.49	0.41	0.83	0.33	0.38	0.37	0.23
4	0.20	0.27	0.57	0.75	0.65	1.17	0.59	0.67	0.61	0.30
5	0.23	0.33	0.90	0.93	0.82	1.40	0.97	1.12	0.84	0.39
6	0.37	0.49	0.92	1.01	0.96	1.50	1.20	1.06	0.94	0.37

Table A66: Fluxes (µg/h) of acetaminophen. 0.07 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.11	0.24	0.26	0.68	0.26	0.39	0.24	0.22
3	0.17	0.30	0.40	0.76	0.67	1.33	0.74	0.78	0.64	0.36
4	0.41	0.65	0.80	1.34	1.13	1.94	1.09	1.15	1.06	0.47
5	0.57	0.87	1.21	1.46	1.44	2.26	1.48	1.97	1.41	0.55
6	0.86	1.15	1.17	2.05	1.63	2.28	1.54	1.49	1.52	0.47

Table A67: Fluxes (µg/h) of naltrexone. 0.07 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.32	0.00	0.00	0.00	0.06	0.07	0.06	0.11
3	0.16	0.00	0.52	0.00	0.00	0.00	0.04	0.00	0.09	0.18
4	0.22	0.00	0.54	0.00	0.00	0.00	0.16	0.17	0.14	0.19
5	0.22	0.00	0.55	0.00	0.00	0.48	0.32	0.38	0.25	0.23
6	0.45	0.00	0.53	0.21	0.25	0.52	0.58	0.41	0.37	0.20

Table A68: Fluxes (µg/h) of buprenorphine. 0.07 mg/ml NTX & 0.5 mg/ml BUP.

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6		Cell 7		Cell 8	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.2	2.9	1.6	2.5	1.8	1.7	3.9	19.4	4.2	19.1	3.9	11.0	0.5	1.5	1.4	2.0
2	3.0	1.4	2.3	1.2	3.0	0.8	5.2	5.6	5.3	3.9	4.7	2.0	0.7	1.5	1.7	1.2
3	3.5	0.7	2.7	0.5	3.5	0.3	5.8	2.8	5.9	1.8	5.4	2.3	0.9	1.0	1.8	1.4
4	3.9	1.1	3.0	0.7	4.0	0.3	6.5	1.8	6.5	1.7	6.1	1.9	1.0	1.0	2.0	1.1
5	4.2	0.4	3.4	0.5	4.5	0.2	7.0	1.5	6.8	0.9	6.7	1.7	1.1	0.8	2.1	1.0
6	4.5	0.5	3.8	0.5	4.8	0.1	7.4	1.1	7.1	1.2	7.2	1.2	1.1	0.6	2.2	0.7
7	5.0	0.6	4.2	0.6	5.5	0.2	7.9	1.3	7.5	1.2	7.6	1.4	1.1	0.5	2.2	0.5
8	5.5	0.4	4.6	0.5	5.9	0.2	8.3	1.1	7.9	1.0	8.0	0.9	1.1	0.4	2.2	0.5
9	5.9	0.4	4.8	0.3	6.4	0.3	8.6	1.1	8.1	1.1	8.4	1.1	1.2	0.4	2.2	0.2
10	6.2	0.3	5.2	0.4	6.8	0.1	8.7	0.6	8.5	0.9	8.6	0.8	1.2	0.3	2.2	0.2
11	7.1	0.3	5.5	0.3	7.1	0.2	9.1	1.0	8.7	0.8	9.0	1.0	1.2	0.3	2.2	0.2
12	7.5	0.3	5.8	0.2	7.5	0.2	9.3	0.6	9.0	0.6	9.5	0.9	1.2	0.3	2.2	0.3
Total BUP in tapes (µg)		9.3		8.2		4.7		38.0		34.2		26.2		8.7		9.3
BUP in viable tissue (µg)		-		-		-		51.6		51.2		53.6		76.9		61.8
NTX in viable tissue (µg)		-		-		-		9.1		9.2		5.6		19.2		9.0

Table A69: Drug in the skin. 0.07 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.12	0.09	0.39	0.16	0.18	0.19	0.12	0.18	0.10
3	0.30	0.27	0.83	0.41	0.41	0.36	0.32	0.41	0.19
4	0.49	0.42	1.09	0.65	0.73	0.58	0.62	0.65	0.22
5	0.59	0.52	1.35	0.83	0.77	0.77	1.02	0.84	0.28
6	0.67	0.57	1.38	1.04	1.08	1.05	1.29	1.01	0.30

Table A70: Fluxes (µg/h) of acetaminophen. 0.14 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.40	0.19	0.66	0.68	1.03	0.97	0.56	0.64	0.29
3	1.26	0.78	1.40	1.77	2.14	1.99	1.72	1.58	0.47
4	1.99	1.50	2.08	2.82	3.46	3.12	2.70	2.53	0.69
5	2.54	1.91	2.68	3.42	3.51	3.32	3.61	3.00	0.63
6	2.44	2.20	2.93	3.92	4.24	4.24	3.64	3.37	0.85

Table A71: Fluxes (µg/h) of naltrexone. 0.14 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.20	0.21	0.44	0.24	0.00	0.16	0.17
5	0.38	0.16	0.56	0.40	0.59	0.80	0.42	0.47	0.20
6	0.44	0.21	0.73	0.67	0.87	0.84	0.50	0.61	0.24

Table A72: Fluxes (µg/h) of buprenorphine. 0.14 mg/ml NTX & 0.5 mg/ml BUP.

	Cell 1		Cell 2		Cell 3		Cell 4		Cell 5		Cell 6		Cell 7	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	3.5	8.73	3.6	8.70	2.1	3.00	1.5	1.84	2.3	5.55	2.0	3.13	0.9	1.89
2	4.7	1.92	4.2	1.04	2.2	0.78	2.0	1.07	2.9	1.77	2.6	1.27	1.2	1.45
3	5.4	0.71	5.3	1.27	2.7	0.45	2.5	1.00	3.5	0.89	3.0	1.28	1.4	1.58
4	5.7	0.68	5.7	0.69	3.2	0.34	3.1	0.66	3.9	1.19	3.5	1.11	1.8	1.25
5	6.2	0.69	6.1	0.59	3.4	0.35	3.5	0.66	4.5	1.93	3.9	0.82	1.9	1.51
6	6.7	0.62	6.6	0.56	4.2	0.34	3.9	0.49	5.2	1.41	4.3	1.24	2.0	0.89
7	7.2	0.70	6.8	0.35	4.5	0.29	4.4	0.54	5.9	0.43	4.7	0.54	2.1	0.73
8	7.4	0.31	7.2	0.34	4.9	0.25	4.7	0.43	6.3	0.86	5.1	0.69	2.2	0.67
9	7.8	0.45	7.5	0.48	5.8	0.21	5.0	0.32	6.7	0.86	5.4	0.48	2.2	0.49
10	8.0	0.18	8.0	0.95	6.2	0.23	5.4	0.36	7.1	0.80	5.7	0.51	2.2	0.51
11	8.5	0.37	8.6	0.91	6.5	0.18	5.7	0.37	7.7	3.03	6.1	0.95	2.2	0.36
12	8.7	0.17	8.8	0.55	6.6	0.18	6.1	0.37	7.9	0.43	6.5	0.32	2.3	0.40
Total BUP in tapes (µg)		15.54		16.43		6.61		8.12		19.14		12.35		11.72
BUP in viable tissue (µg)		59.2		77.7		23.9		42.2		35.5		34.2		102.9
NTX in viable tissue (µg)		13.2		19.2		7.8		10.5		6.7		7.0		11.9

Table A73: Drug in the skin. 0.14 mg/ml NTX & 0.5 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.01	0.03
2	0.16	0.11	0.10	0.00	0.12	0.20	0.27	0.52	0.21	0.19	0.15
3	0.29	0.25	0.28	0.17	0.28	0.43	0.45	0.85	0.41	0.38	0.20
4	0.39	0.34	0.39	0.24	0.44	0.52	0.58	1.04	0.58	0.50	0.23
5	0.42	0.60	0.50	0.38	0.49	0.65	0.69	1.08	0.81	0.62	0.22
6	0.48	0.61	0.56	0.37	0.56	0.53	0.73	1.26	0.65	0.64	0.25

Table A74: Fluxes (µg/h) of acetaminophen. 0.14 mg/ml NTX & 1.0 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.43	0.59	0.31	0.26	0.36	0.43	0.41	0.48	0.32	0.40	0.10
3	1.13	1.55	1.18	0.94	1.14	1.46	0.89	0.88	0.79	1.11	0.26
4	1.81	2.30	1.75	1.37	1.65	1.90	1.17	1.23	1.12	1.59	0.40
5	2.16	3.44	2.27	1.86	2.15	2.42	1.36	1.36	1.42	2.05	0.67
6	2.52	3.47	2.63	2.32	2.33	2.18	1.38	1.64	1.20	2.18	0.70

Table A75: Fluxes (µg/h) of naltrexone. 0.14 mg/ml NTX & 1.0 mg/ml BUP.

Hours	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Mean	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.17	0.11	0.12	0.00	0.00	0.00	0.12	0.00	0.00	0.06	0.07
5	0.34	0.36	0.25	0.00	0.00	0.27	0.20	0.20	0.09	0.19	0.13
6	0.49	0.60	0.49	0.14	0.29	0.26	0.25	0.18	0.06	0.31	0.18

Table A76: Fluxes (µg/h) of buprenorphine. 0.14 mg/ml NTX & 1.0 mg/ml BUP.

	Cell 7		Cell 8		Cell 9	
Tape	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)	Cumulative depth (µm)	Mass of BUP (µg)
1	2.7	12.6	2.7	23.7	2.2	14.0
2	3.7	13.8	3.9	6.4	3.2	5.8
3	4.2	3.4	4.5	5.0	4.1	5.3
4	4.6	3.4	5.1	4.8	4.5	8.4
5	4.9	2.3	5.7	5.1	4.6	3.5
6	5.3	2.2	6.3	3.6	4.8	2.6
7	5.6	2.1	6.9	2.4	5.0	1.3
8	5.8	2.0	7.3	1.3	5.3	2.5
9	6.1	1.5	7.7	2.7	5.5	2.0
10	6.3	2.1	8.2	1.9	5.8	2.9
11	6.5	0.8	8.6	5.0	6.1	2.4
12	6.6	0.9	8.7	2.4	6.3	1.3
Total BUP in tapes (µg)		47.1		64.2		51.9
BUP in viable tissue (µg)		96.5		102.8		108.0
NTX in viable tissue (µg)		9.7		9.5		11.8

Table A77: Drug in the skin. 0.14 mg/ml NTX & 1.0 mg/ml BUP.